

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61N 1/32	A1	(11) International Publication Number: WO 99/01175 (43) International Publication Date: 14 January 1999 (14.01.99)														
<p>(21) International Application Number: PCT/EP98/03976</p> <p>(22) International Filing Date: 30 June 1998 (30.06.98)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>97/08233</td> <td>30 June 1997 (30.06.97)</td> <td>FR</td> </tr> <tr> <td>97/08232</td> <td>30 June 1997 (30.06.97)</td> <td>FR</td> </tr> <tr> <td>60/067,488</td> <td>1 December 1997 (01.12.97)</td> <td>US</td> </tr> <tr> <td>60/067,487</td> <td>1 December 1997 (01.12.97)</td> <td>US</td> </tr> <tr> <td>60/083,858</td> <td>1 May 1998 (01.05.98)</td> <td>US</td> </tr> </table> <p>(71) Applicants (for all designated States except US): RHONE-POULENC RORER S.A. [FR/FR]; 20, avenue Raymond Aron, F-92160 Antony (FR). INSTITUT GUSTAVE ROUSSY [FR/FR]; 39, rue Camille Desmoulins, F-94805 Villejuif (FR). CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): BUREAU, Michel [FR/FR]; 1, square Sainte Clothilde, F-92210 Saint Cloud (FR). MIR, Lluis [FR/FR]; 22, allée des Vaupépins, F-91370 Verrières Le Buisson (FR). SCHERMAN, Daniel [FR/FR]; 10, rue Erard, F-75012 Paris (FR). SCHWARTZ,</p>	97/08233	30 June 1997 (30.06.97)	FR	97/08232	30 June 1997 (30.06.97)	FR	60/067,488	1 December 1997 (01.12.97)	US	60/067,487	1 December 1997 (01.12.97)	US	60/083,858	1 May 1998 (01.05.98)	US	<p>Bertrand [FR/FR]; 13-15, rue Paul Bert, F-94700 Maisons Alfort (FR).</p> <p>(74) Agent: DERNONCOUR, Roxane; Rhône-Poulenc Rorer S.A., Direction Brevets, 20, avenue Raymond Aron, F-92165 Antony Cedex (FR).</p> <p>(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, JP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
97/08233	30 June 1997 (30.06.97)	FR														
97/08232	30 June 1997 (30.06.97)	FR														
60/067,488	1 December 1997 (01.12.97)	US														
60/067,487	1 December 1997 (01.12.97)	US														
60/083,858	1 May 1998 (01.05.98)	US														
<p>(54) Title: DEVICE FOR OPTIMIZED ELECTROTRANSFER OF NUCLEIC ACID VECTORS TO TISSUES IN VIVO</p> <p>(57) Abstract</p> <p>This invention is directed to systems and devices that provide for remarkable enhancement of <i>in vivo</i> transfer into cells, particularly muscle cells and tumor cells, of nucleic acid vectors using weak electric fields, to increase the efficiency of such transfers. The devices of the invention are designed to provide an optimum voltage gradient to enhance migration of nucleic acid vectors into cells, without damaging the cells or tissue. Such devices are characterized by unique arrangements of electrodes, and by unique power limits defined by maximum voltage settings.</p>																

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

APPARATUS FOR OPTIMIZED ELETROTRANSFER OF NUCLEIC ACID VECTORS TO TISSUES *IN VIVO*

5

FIELD OF THE INVENTION

This invention is directed to remarkable enhancement of *in vivo* transfer into cells, particularly muscle cells, of nucleic acid vectors using weak electric fields, to increase the efficiency of such transfers. The invention specifically relates to methods, devices, and compositions that effect such nucleic acid vector transfer for gene therapy. The devices of the invention are designed to provide an optimum voltage gradient to enhance migration of nucleic acid vectors into cells, without damaging the cells or tissue. Such devices are characterized by unique arrangements of electrodes, and by unique power limits defined by maximum voltage settings.

15

BACKGROUND OF THE INVENTION

Vectors and Methods for Gene Delivery

The transfer of genes into a given cell is at the root of gene therapy. However, one of the problems is to introduce a sufficient quantity of nucleic acid into the host cells to be treated. The gene of interest must be expressed in transfected cells. One of the approaches adopted in this connection has been the integration of nucleic acid in viral vectors, particularly in retroviruses, adenoviruses, or adeno-associated viruses. These systems take advantage of the cell penetration mechanisms developed by the viruses, as well as their protection against degradation. However, this approach presents problems. One is a risk of production of infectious viral particles susceptible to dissemination in the host organism and, in the case of retroviral vectors, a risk of insertional mutagenesis. Furthermore, the insertion capacity of a therapeutic or vaccine gene in a viral genome remains limited. Finally, immune responses are often generated against viral vectors, which makes re-administration of the virus ineffective because of immune clearance, and may also result in inflammation.

Another approach (Wolf et al., Science 247, 1465-68, 1990; Davis et al., Proc. Natl. Acad. Sci. USA 93, 7213-18, 1996; US Patent No. 5,580,859 to Felgner et al.) involves administering into the muscle or blood stream a plasmid-type nucleic acid, whether linked or not to compounds intended to facilitate its transfection, like proteins, liposomes, charged lipids or cationic polymers, such as polyethylenimine, which are good *in vitro* transfection agents (Behr et al., Proc. Natl. Acad. Sci. USA 86, 6982-6, 1989; Felgner et al., Proc. Natl. Acad. Sci. USA 84, 7413-7, 1987; Boussif et al., Proc. Natl. Acad. Sci. USA 92, 7297-301, 1995; US Patent

5,676,954 and EP Patent 425 475).

As far as the muscle is concerned, since the original publication of Wolff et al., *supra*, showing the capacity of the muscular tissue to incorporate DNA injected in free plasmid form, numerous investigators have tried to enhance this process (Manthorpe et al., 1993, Human Gene Ther. 4, 419-431; Wolff et al., 1991, BioTechniques 11, 474-485). Certain trends have emerged from these tests, notably:

- the use of mechanical solutions to force entry of DNA into the cells, by adsorbing the DNA on balls then propelled on the tissues ("gene gun") (Sanders Williams et al., 1991, Proc. Natl. Acad. Sci. USA 88, 2726-2730; Fynan et al., 1993, BioTechniques 11, 474-485).

10 These processes have proven effective in vaccination strategies, but touch only the superficial tissue layers. In the case of the muscle, their use would necessitate a surgical approach in order to afford access to the muscles, for the particles do not cross the cutaneous tissues;

- the injection of DNA, no longer in free plasmid form, but linked to molecules capable of serving as vehicle facilitating entry of the complexes into the cells. The cationic lipids used in numerous other transfection processes have up to now turned out to be disappointing in their application to muscle, for those which have been tested have proven to inhibit transfection (Schwartz et al., 1996, Gene Ther., 405-411). The same is true for cationic peptides and polymers (Manthorpe et al., 1993, Human Gene Ther. 4, 419-431). The only case of favorable combination seems to be the polyvinyl alcohol or polyvinylpyrrolidone mixture with DNA. The resulting increase of these combinations represents only a factor of less than 10 in relation to the bare injected DNA (Mumper et al., 1996, Pharmaceutical Research 13, 701-709);

- pretreatment of the tissue to be injected with solutions intended to enhance DNA diffusion and/or stability (Davis et al., 1993, Hum. Gene Ther. 4, 151-159) or to favor the entry of nucleic acids, e.g., the induction of cell multiplication or regeneration phenomena. The treatments have concerned, in particular, the use of local anesthetics or cardiotoxin, vasoconstrictors, endotoxin or other molecules (Manthorpe et al., 1993, Human Gene Ther. 4, 419-431; Danko et al., 1994, Gene Ther. 1, 114-121; Vitadello et al., 1994, Hum. Gene Ther. 5, 11-18). These pretreatment protocols are difficult to manage. Bupivacaine, in particular, must be used at very close to lethal doses in order to be effective. The pre-injection of hyperosmotic sucrose, intended to enhance diffusion, does not increase the level of transfection in the muscle (Davis et al., 1993).

Other tissues have been transfected *in vivo* either by using plasmid DNA alone, or by

linkage to synthetic vectors (reviews of Cotten and Wagner (1994), Current Opinion in Biotechnology 4, 705; Gao and Huang (1995), Gene Therapy, 2, 710; Ledley (1995), Human Gene Therapy 6, 1129). The principal tissues studied were the liver, the respiratory epithelium, the wall of vessels, the central nervous system and tumors. In all these tissues the transgene
5 expression levels proved too low to foresee a therapeutic application (for example, in the liver, Chao et al. (1996), Human Gene Therapy 7, 901), although some encouraging results have recently been presented for plasmid DNA transfer in the vascular wall (Iires et al. (1996), Human Gene Therapy 7, 959 and 989). In the brain the effectiveness of transfer is very slight, as it is in tumors (Schwartz et al. 1996, Gene Therapy 3, 405; Lu et al., 1994, Cancer Gene
10 Therapy 1, 245; Son et al., Proc. Natl. Acad. Sci. USA 91, 12669).

Electroporation and Iontophoresis for Gene Delivery

Electroporation, or the use of electric fields to permeabilize cells, is commonly used *in vitro* to promote DNA transfection in culture cells. This phenomenon depends on achieving a
15 threshold electric field strength. Electroporation was observed at electric fields of relative high intensity, in the order of 800 to 1,200 volts/cm for animal cells. This technique was also proposed *in vivo* in order to enhance the efficacy of antitumoral agents, like bleomycin, in solid tumors in man (US patent No. 5,468,228, L.M. Mir). With pulses of very short duration (100 microseconds), these electric conditions (800 to 1,200 volts/cm) are very well adapted to
20 the intracellular transfer of small molecules. These conditions (pulses of 100 microseconds) were applied without enhancement for *in vivo* nucleic acid transfer into the liver, where fields below 1,000 volts/cm proved totally ineffective and even inhibitory compared to DNA injection in the absence of electric pulses (patent WO 97/07826 and Heller et al., FEBS Letters, 389, 225-8, 1996).

25 That technique presents difficulties, moreover, for *in vivo* application, for the administration of fields of such intensity can cause extensive tissue lesions. Lesions of target tissues are not a problem in the treatment of tumors in cancer patients, but can represent a major disadvantage when nucleic acid is administered into tissues other than tumor tissues and, in particular, into the striated muscle.

30 There are three basic types of systems utilized to administer electric pulses for electroporation and iontophoresis: external electrodes, internal electrodes (including catheters), and combined external and internal electrodes.

External Electrodes

In one type of device, electrodes are positioned externally with respect to the patient. See, for example, US Patent Nos. 5,318,514 to Hofmann; 5,439,440 to Hofmann; 5,462,520 to Hofmann; 5,464,386 to Hofmann; 5,688,233 to Hofmann et al.; and 5,019,034 to Weaver et al.;
5 the disclosures of which are incorporated herein by reference. With an external electrode device, the electrodes are in contact with a surface tissue region of a patient. The device can be used non-invasively by applying the electrodes to the skin of the patient or invasively by applying the electrodes to the surface of an organ that has been exposed surgically.

The Hofmann '514 patent discloses a device that is used to implant macromolecules such
10 as genes, DNA or pharmaceuticals into a preselected surface tissue region of a patient. The device has a head assembly which includes, in a first embodiment, a serpentine conductor positioned on an open-pore elastomer, both of which are carried on a generally planar support member. Adjacent parallel segments of the serpentine conductor serve as electrodes. To administer electric pulses to the patient, the head assembly is placed in contact with the
15 preselected surface tissue region of the patient, placing the conductor in contact with the skin. A liquid medium carrying the macromolecules is transferred to the skin of the patient by delivery to the elastomer of the liquid which is absorbed or soaked up by the elastomer. A switch is then engaged to deliver a high voltage pulse from a signal generator to the electrodes, causing an electric field to be generated across the electrodes. The depth of the electric field into the skin is
20 proportional to the gap between the electrodes. The electric field injects the liquid into the tissue region.

In an alternative embodiment, the head assembly includes a plurality of fine needles extending generally perpendicular to the planar support member. The needles are arranged in rows and are connected alternatively to the output of the signal generator so that each needle is
25 adjacent another needle of opposite polarity. The needles penetrate the outer most layers of skin cells and facilitate the administration of the electric pulses into the target area.

The Hofmann '440 patent discloses an apparatus which includes adjustably spaced electrodes for generating an electric field. The electrodes are mounted on a moveable linkage so that the electrodes can be manipulated by the user to move toward and away from one another like
30 the jaws of a clamp. In operation, the electrode jaws are opened and a selected tissue to be treated is gripped between the electrode jaws. A signal generator connected to the electrodes is operated by a suitable switching device to generate the electric fields in the tissue between the electrodes.

Internal Electrodes

A second type of electrotransfer system utilizes implantable or insertable electrodes which are placed inside the patient to deliver an electric field to the area adjacent to the implanted/inserted electrode. See, for example, US Patent Nos. 5,304,120 to Crandell et al.;
5 5,507,724 to Hofmann et al.; 5,501,662 to Hofmann; 5,702,359 to Hofmann et al.; and 5,273,525 to Hofmann; the disclosures of which are incorporated herein by reference.

The Crandell '120 patent discloses a catheter that is inserted into a selected blood vessel of a patient. The catheter includes a plurality of axially extending, circumferentially spaced electrodes that are placed in contact with the inner wall of the blood vessel. A liquid medium
10 containing the macromolecules is then infused into the blood vessel adjacent the electrodes and the electrodes are energized to apply the predetermined electrical signal for electrotransfer. The spaced electrodes can be serpentine or parallel strips which are energized to create the desired electric field.

The Hofmann '724 patent is another example of a catheter-based electrotransfer device
15 having spaced apart electrodes which are positioned on the outside of a catheter that is inserted into a blood vessel to contact the wall of the vessel to be treated.

The Hofmann '662 patent discloses a pair of spaced apart electrodes mounted within a cylindrical dielectric carrier. The electrodes are positioned around the center of the blood vessel a predetermined uniform distance apart from each other and near the center of the vessel so that
20 blood flowing in the vessel passes between the electrodes. The cylindrical dielectric carrier is implanted surgically within a surrounding blood vessel. A predetermined electrical signal is applied to the electrodes to create electric fields in the blood flowing between the electrodes.

The Hofmann '525 patent discloses a dual-needle syringe in which the needles serve as the electrodes for carrying out the electrotransfer. Once the needles have been inserted into the target
25 area, an electrical signal is applied to the electrodes to direct the electric field to the target area. The Hofmann '359 patent discloses also needle-based electrodes used for electrotransfer.

Combined External and Internal Electrodes

A third type of electrotransfer device combines features of the above-mentioned systems.
30 These devices utilize at least one internally placed electrode and at least one externally placed electrode to deliver the electric field to the desired tissue area. See, for example, US Patent No. 5,286,254 to Shapland et al.; 5,499,971 to Shapland et al.; 5,498,238 to Shapland et al.; 5,282,785 to Shapland et al.; and 5,628,730 to Shapland et al.; the disclosures of which are

incorporated herein by reference.

Typical of these devices is the one described in the Shapland '785 patent which discloses a catheter having a drug chamber with a drug delivery wall (for example, a wall made of permeable or semipermeable material which can pass drugs or other macromolecules therethrough) and an electrode located inside of the catheter in an opposed relation to the drug delivery wall. A second electrode is located at a remote site on a patient's skin. A liquid containing desired macromolecules is delivered to the drug chamber to be placed in the electric field generated between the two electrodes when they are provided with current. In this manner, the macromolecules are delivered to the target area.

Other features are disclosed in the above-mentioned patents, including: reversing the polarity of the electrodes to drive excess macromolecules in a direction opposite to that used for delivery (for example, the Shapland '238 and the Shapland '785 patents); systems for synchronizing the delivery of the current to the electrodes with the ventricular depolarization of the heart to avoid electrically induced arrhythmias or unnatural heart rhythms (for example, US Patent Nos. 5,236,413 and 5,425,703 to Feiring and Patent No. 5,634,899 to Shapland); and using an ultrasonic piezoelectric transducer instead of electrodes to generate sound waves as the driving force for macromolecule delivery, known as phonophoresis (for example, the Shapland '238 and the Shapland '730 patents).

SUMMARY OF THE INVENTION

While all the studies cited mention the need for elevated electric fields, in the order of 1,000 volts/cm, to be effective *in vivo*, most unexpectedly and remarkably the applicants have now shown that *in vivo* nucleic acid transfer into tissues is very substantially increased without untoward effects, by subjecting the tissue to electric pulses of low intensity (less than 600 volts/cm), *e.g.*, of 100 to 200 volts/cm, and of relatively long duration. Furthermore, the applicants discovered that the wide variability of plasmid-carried transgene expression observed in the prior art of DNA transfer into the muscle was notably reduced by the process according to the invention.

Accordingly, this invention concerns a process and device for nucleic acid transfer into tissues *in vivo*, *e.g.*, one or more striated muscles or tumors, in which the tissue cells are brought in contact with the nucleic acid to be transferred by direct administration into the tissue or by topical or systemic administration, and in which transfer is ensured by application to the tissues of one or more electric pulses of intensity ranging between 1 and 400 volts/cm for muscle, and 1 and 600 volts/cm for tissues such as tumors.

Accordingly, the present invention provides a system, such as an improved apparatus, for *in vivo* nucleic acid transfer into cells of multicellular eukaryotic organisms, in which the tissue cells are brought in contact with the nucleic acid to be transferred by direct administration into the tissue or by topical or systemic administration, and in which transfer is ensured by application to the tissue of one or more electric pulses delivered by an apparatus of the invention set to provide the specified intensity. In particular, the electric field strength can range between 1 and 600 volts/cm for delivery of a nucleic acid to tumor cells, and between 1 and 400 volts/cm for delivery of a nucleic acid to muscle cells. The system (or apparatus) of the invention comprises an electrical pulse generator (or means for generating an electrical pulse), wherein the electrical pulse generator produces electric pulses with pulse times of greater than 1 millisecond and of intensity ranging between 1 and 400 or 600 volts/cm at a frequency of between 0.1 and 1000 Hz; and electrodes connected to the electrical pulse generator for generating an electric field in a tissue *in vivo* in contact with the electrodes. In a specific embodiment, the electrical pulse generator produces pulse of intensity ranging between 30 and 300 volts/cm (for transfer into muscle) and between 400 and 600 V/cm for transfer into tumor cells and other small cells. In another specific embodiment, the electrical pulse generator produces pulse times of greater than 10 milliseconds. In still another specific embodiment, the electrical pulse generator produces between 2 and 1000 pulses.

According to the invention, the system or improved apparatus electrical pulse generator can produce pulses irregularly in relation to one another, whereby a function describing the intensity of the field dependent on time of an pulse is variable, with the proviso that at no time does the system or apparatus supply an electric field greater (or less) than the parameters set forth above. For example, the integral of the function describing variation of the electric field with time can exceed 1 kV•msec/cm; and in a further embodiment exceeds or is equal to 5 kV•msec/cm.

The electrical pulse generator (pulse generating means) can produce pulses selected from the group consisting of square-wave pulses, exponential decay waves, oscillating unipolar waves of limited duration, and oscillating bipolar waves of limited duration. Preferably, the electrical pulse generator produces square-wave pulses.

Various electrode configurations are contemplated by the invention. For example, the electrode can be an external electrode for placement on a tissue to be treated, *e.g.*, for transferring nucleic acids into cells of a surface tissue of a subject. Alternatively, the electrode can be an internal electrode or tissue penetrating electrode, which is implantable in a tissue to be treated. Such an internal electrode can be a needle, and may be configured as an injector system

making possible the simultaneous administration of nucleic acids and of the electric field. In another embodiment, the invention provides both an external electrode and an internal electrode.

An external electrode of the invention can be dimensioned to contact an external portion of a subjects body in close proximity to a large muscle. In a specific embodiment, such
5 an electrode is a flat plate electrode; in another embodiment, it is a semi-cylindrical plate electrode.

In still another embodiment, the an electrode is an intra-arterial or intravenous electrode, for example a flexible catheter apparatus modified according to the invention.

The preferred material for an electrode of the invention is stainless steel.

10 The improved apparatus of the invention can be produced by modifying prior art devices, and particularly the means for generating an electric field of such devices, to generate an electric field of the invention. For example, the means for generating an electric pulse can be adapted to produce pulses ranging between 1 and 400 or 600 volts/cm by modifying the voltage
gate not to exceed a voltage corresponding to 400 or 600 volts/cm. In a specific embodiment of
15 such a modified device, the voltage can be set at a constant voltage and the electrodes can be set at a constant spacing distance. Alternatively, the means for generating an electric pulse can be adapted to produce pulses ranging between 1 and 400 or 600 volts/cm by labeling the device not to exceed a voltage corresponding to 400 or 600 volts/cm.

Thus, an object of the invention is to provide a system, or improve existing devices, to
20 supply an electric field having a voltage gradient, pulse width, and number of pulses that have been found to be optimum for transfer of nucleic acids without damaging tissue.

A particular object of the invention is to provide for electrotransfer of nucleic acids under milder and less damaging conditions that are used for electroporation (with voltage gradients exceeding 600, and usually exceeding 1000, volts/cm).

25 Still another particular object of the invention is to provide for much more effective intracellular delivery of nucleic acids than can be achieved under the very low strength electric fields employed for iontophoresis.

Yet another advantage of the invention is to provide for efficient, reproducible delivery of nucleic acids to muscle cells.

30 These and other objects of the invention have been achieved as set forth above, and as described in greater detail in the Detailed Description of the Invention, as well as in the Examples with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1: Effects of electric pulses of high field intensity on the transfection of plasmid DNA pXL2774 in the cranial tibial muscle in the mouse; mean values \pm SEM.

FIGURE 2: Effects of electric pulses of intermediate field intensity on the transfection of plasmid DNA pXL2774 in the cranial tibial muscle in the mouse; mean values \pm SEM.

5 FIGURE 3: Effects of electric pulses of weak field intensity and of different duration on the transfection of plasmid DNA pXL2774 in the cranial tibial muscle in the mouse; mean values \pm SEM.

FIGURE 4: Effects of electric pulses of weak field intensity and of different duration on the transfection of plasmid DNA pXL2774 in the cranial tibial muscle in the mouse; mean
10 values \pm SEM.

FIGURE 5: Effectiveness of electrotransfer of plasmid DNA pXL2774 in the cranial tibial muscle of the mouse at low electric field intensities; mean values \pm SEM.

FIGURE 6: Kinetics of expression of luciferase in mouse tibial cranial muscle. Administration of plasmid pXL2774 with (■) and without (X) electrotransfer; average values \pm
15 SEM.

FIGURE 7: Level of expression as a function of the dose of DNA administered with (●) and without (□) electrotransfer.

FIGURE 8: Effect of different types of electrodes on electrotransfer efficiency.

FIGURE 9: Kinetics of serum concentration of secreted alkaline phosphate. Administration
20 of plasmid pXL3010 with (■) et without (◆) electrotransfer; average values \pm SEM.

FIGURE 10: Kinetics of expression of aFGF in muscle with (open histogram bars) or without (solid histogram bars) electrotransfer.

FIGURE 11: Map of plasmids pXL3179 and pXL3212.

FIGURE 12: Map of plasmids pXL3388 and pXL3031.

25 FIGURE 13: Map of plasmids pXL3004 and pXL3010.

FIGURE 14: Map of plasmids pXL3149 and pXL3096.

FIGURE 15: Map of plasmids pXL3353 and pXL3354.

FIGURE 16: Map of plasmid pXL3348.

30

DETAILED DESCRIPTION OF THE INVENTION

As pointed out above, the present invention provides greatly enhanced *in vivo* nucleic acid transfer into tissues by subjecting the tissue to electric pulses of low intensity. For example, electric fields of less than 600 volts/cm have been found to enhance nucleic acid
35 transfer into tumors, and less than 400 volts/cm, and preferably, 100 to 200 volts/cm for

electrodes placed about 0.5 to 1 cm apart in muscle. These fields are applied for relatively long duration. Furthermore, the applicants discovered that the wide variability of transgene expression observed in the prior art of DNA transfer into the muscle was notably reduced by the process according to the invention. Finally, it has been discovered that expression persists for a long period of time, *e.g.*, greater than 60 days. In a specific example, high level expression was detected 63 days.

As can be readily determined from the description provided herein, Applicants have termed transfer of nucleic acids into cells *in vivo* under these conditions "electrotransfer"; an appropriate alternative term used herein is "electrotransfection". Both of these terms distinguish the optimized conditions for nucleic acid transfer from "electroporation" (using electric fields greater than 800 V/cm) and iontophoresis (using very low strength electric fields).

Accordingly, this invention concerns processes, systems and devices (or apparatus), and compositions for *in vivo* nucleic acid transfer into tissues, particularly striated muscle, in which the tissue cells are brought in contact with the nucleic acid to be transferred by direct administration into the tissue or by topical or systemic administration, and in which transfer is ensured by application to said tissues of one or more electric pulses of intensity ranging between 1 and 600 volts/cm (*e.g.* for tumor cells) or between 1 and 400 volts/cm for muscle cells. In other words, the present invention particularly concerns systems (*i.e.*, devices or apparatus) for electrotransfer.

According to one preferred embodiment, the process of the invention is applied to tissues whose cells have particular geometries, like, for example, cells of large size and/or of elongated shape and/or naturally responding to electric potentials and/or having a specific morphology.

The intensity of the field preferably ranges between 4 and 400 volts/cm for muscle, and up to 600 volts/cm for tumors, and the total duration of application exceeds 1 millisecond (msec), and preferably 10 msec. In specific examples, the total duration is 8 msec or longer. In a number of examples, the pulse duration is 20 msec, and durations greater than 40 msec were found to be effective. The number of pulses used, is, for example, from 1 to 1,000 pulses, preferably 2 to 100 and more preferably 4 to 20, and the pulse frequency ranges between 0.1 and 1,000 hertz (Hz); more precisely between 0.2 and 100 Hz. In specific embodiments, frequencies of 2, 3, and 4 Hz were found to be effective. The pulses can also be delivered irregularly and the function describing the intensity of the field dependent on time can be variable. The integral of the function describing the variation of the electric field with time is greater than 1 kV•msec/cm. According to a preferred embodiment of the invention, that integral is higher than

or equal to 5 kV•msec/cm. However, it should be noted, as one of ordinary skill in the art can readily appreciate, that this integrated function must be achieved at the sub-electrophoretic voltages described *supra*.

5 In a specific example, which represents a departure from the invention, the device of the invention can supply a combination of at least one higher voltage pulse (greater than 400 V/cm, and preferably between 500 and 800 V/cm) for short duration (less than 1 msec), followed by one or more longer pulses (greater than 1 msec) at much lower electric field strength (less than 200 V/cm).

10 According to one preferred embodiment of the invention, the field intensity of the pulses ranges between 30 and 300 volts/cm.

The electric pulses are chosen among square-wave pulses, the electric fields generating exponential decay waves, oscillating unipolar waves of limited duration, oscillating bipolar waves of limited duration or other wave-forms. According to a preferred embodiment of the invention, the electric pulses are square wave pulses.

15 The administration of electric pulses can be carried out by any method known in the art, *e.g.*:

- system of external electrodes placed on both sides of the tissue to be treated, notably, noninvasive electrodes placed in contact with the skin,
- system of electrodes implanted in the tissues,
- 20 • electrodes/injector system making possible the simultaneous administration of nucleic acids and of the electric field.

For administration being carried out *in vivo*, it is sometimes necessary to resort to intermediate products ensuring electric continuity with noninvasive external electrodes. It will, for example, involve an electrolyte in gel form. Examples of appropriate gels include gels used
25 in the Examples, *infra*, as well as gels commonly used in medicine to enhance electrical contacts, such as for electrocardiograms or defibrillators.

Nucleic acids can be administered by any appropriate means, but are preferably injected *in vivo* directly into the tissues or administered by another route, local or systemic, which makes them available on the site of application of the electric field. The nucleic acids can be
30 administered with agents permitting or facilitating transfer, as previously mentioned. Those nucleic acids can, notably, be free in solution or linked to synthetic agents or carried by viral vectors. The synthetic agents can be lipids or polymers known to the expert, or even targeting elements making possible fixation on the membrane of the target tissues. Among those elements, vectors carrying sugars, peptides, antibodies, receptors, and ligands, can be

mentioned.

It is conceivable, under these conditions of the invention, that the administration of nucleic acids can precede, be simultaneous or even follow the application of electric fields, provided of course that the electric field continues to be applied after the nucleic acid is

5 administered.

This invention also concerns a nucleic acid and an electric field of intensity ranging between 1 and 600 volts/cm (preferably 400 volts/cm), as a combination product for their simultaneous, separate or time-staggered *in vivo* administration into mammal cells and, in particular, into human cells. The intensity of the field preferably ranges between 4 and 400
10 volts/cm and, even more preferably, the field intensity ranges between 30 and 300 volts/cm for transfer into muscle. For transfer into tumors and cells with similar electrotransfer reception properties, a preferred electric field intensity is 400-600 V/cm; preferably about 500 (*i.e.*, $500 \pm 10\%$, preferably 5%) V/cm. As can be readily appreciated by one of ordinary skill in the art, such a combination defines a nucleic acid structure, in which the nucleic acid adopts a specific
15 orientation relative to the electric field, as well as specific secondary and tertiary structure in the presence of the electric field. In addition, the DNA will be associated with the extracellular components found in the target tissue, which is in distinction to DNA undergoing low field electrophoresis in an agarose gel or other laboratory conditions.

20

Electrotransfer Systems and Apparatus

The primary components of any electrotransfer system (*i.e.*, apparatus or device; the terms are used herein interchangeably) consist of an electric pulse generator that is designed or modified to provide pulses of no more than 600 V/cm, and electrodes. A system or apparatus of the invention for delivery of a nucleic acid specifically to muscle provides pulses of no more
25 than 400 volts/cm. Naturally, the actual voltage will depend on the distance between the electrodes. As is well known in the art, this distance affects the specific resistance (resistivity) through the target tissue. Accordingly, the actual voltage applied will depend on the resistance so that current, and thus the total power, is kept within acceptable levels. The term "acceptable levels" as used herein means that the total power does not result in irreversible tissue damage,
30 particularly tissue burning. Thus, in a preferred aspect, the apparatus of the invention either controls for acceptable current by fixing voltage and electrode distance, or includes a feedback means to prevent applying too high a voltage for the distance between the electrodes, and thus too much current. A system of the invention may include an oscilloscope or other metering device to monitor voltage, current, or both.

In one embodiment, the system of the invention is prepared using commercially available equipment. Preferably, such equipment is modified to provide the specific electrotransfer conditions defined herein as optimal. In another embodiment, a new apparatus is designed and built to achieve the objectives of the invention. Design specifications of the modified or built pulse generator include, but are by no means limited to, incorporation of a mechanical or electrical controller to maintain the desired voltage gradient, *i.e.*, less than 600 or 400 V/cm, and preferably less than 200 V/cm for administration to muscle. A mechanical control could include, for example, a stop on the voltage selection dial that prevents selection of a voltage that would yield too high an electric field. Alternatively, the device can be built or modified to that such voltages cannot be selected. In still another embodiment, the device can include a breaker or fuse that will trip when the voltage (and thus current) exceeds the parameters of the invention. In yet another embodiment, microprocessor controls can prevent or override selection of too great a voltage. In still another embodiment, a pulse generator is modified simply by applying a label directing use of a particular voltage range that provides the electric field strength of the invention. All of these modifications are routine in the art, and employ standard electrical and mechanical technologies.

As mentioned above, the actual voltage delivered by a system of the invention to achieve the electric field strength defined herein as optimal will depend, in part, on the electrode spacing. If the electrodes are spaced apart in a fixed manner, then the voltage (for a defined tissue, *e.g.*, muscle, liver, heart, or a tumor) may be a pre-defined constant. However, if it is desirable to provided for varying the spacing of the electrodes, then the voltage may have to be adjusted to maintain a constant voltage gradient. This can be determined by measuring the distance between the electrodes, by including measuring means on the electrodes that provides a value for their spacing after adjustment, or by automated measuring means that feedback to the pulse generator to automatically provide the correct voltage (see US Patent 5,439,440 to Hofmann).

The following sections more fully describe pulse generators, electrodes, and devices of the prior art that can be modified in accordance with the invention.

30

Pulse Generators

Pulse generators (also termed "voltage generators" and "pulse or voltage generating means") are electrical devices that produce a current of defined voltage, duration, pulse width, duty cycle (the total time of the pulsing and resting), and pulse frequency. Such devices are well known in the art, and include commercially available pulse generators such as the ELECTRO

CELL MANIPULATOR Model ECM 600, T800L and T820 voltage generators available from the BTX Instruments Division of Genetronics, Inc. of San Diego, California, *e.g.*, as described in US Patent No. 5,704,908, which is incorporated herein by reference in its entirety.

Alternatively, the pulse generator can be an Electropulsator PS 15, available from Jouan, France, as disclosed in the Examples, *infra*. In still another embodiment, a voltage generator that can produce one or more of the wave forms described in US Patent No. 5,634,899, which is incorporated herein by reference in its entirety, can be used. The voltage can be designed to generate pulses of variable shape, intensity, and duration. For example, a pulse of 200 V/cm or 400 V/cm, 5-20 msec, could be followed by a lower intensity, longer pulse. The device could further supply iontophoretic electric fields in combination with electric fields of the invention.

A pulse generator of the invention will have the following specifications:

- generate voltage gradients of between 1 and 600 or 400 V/cm; preferably between 4 and 400 V/cm; more preferably between 30 and 300 V/cm. Specific voltage gradients contemplated for a device of the invention for electrotransfer into muscle are about 100 V/cm and 200 V/cm; preferably less than 200 V/cm. For electrotransfer into tumor cells or similar cells, an electric field of 400-600 V/cm, and optimally about 500 V/cm, has unexpectedly been found to be preferred.
- pulse frequencies of 0.1 to 1,000 hertz (Hz); in a specific embodiment, the frequency is about 2 Hz or greater, up to 10 Hz; preferably greater than 1 Hz. In a preferred embodiment, the frequency is 3 or 4 Hz.
- pulse time (duration) greater than 1 millisecond (msec), with variable duty times; preferably the pulse time is greater than 5 msec; more preferably greater than 10 msec; and more preferably still greater than 20 msec.

In a preferred aspect, the pulse generator of the invention produces at least two, and preferably four, six or eight pulses. It can produce, for example, between 8 and 1,000 pulses. The pulse generator should permit an override or cut-off if the patient begins to experience an adverse event or the electric field strength is out of control. Such an override could be manual or automatic, or both.

It should be apparent to one of the ordinary skill in the art that pulses can be generated by an external signal, such as another device, a computer, etc. For example, for delivery of a nucleic acid to cardiac tissue, the pulse generator will optimally interface with the subject's electrocardiogram so that pulses are synchronized with the heart beat. Such a system preferably includes active pacing of the subject's cardiac rhythm, *e.g.* with a pacemaker (see US Patent No. 5,634,899 to Shapland).

Electrodes

The electrodes of the invention provide the electric field in the tissue. One electrode, the cathode, is negatively charged; the anode is positively charged. Generally, in accordance
5 with the inventions, there is a net flow or flux of ions from one electrode to the other (the flow depends, of course, on the net charge of the ionic species and the polarization of the electrode). In general, nucleic acids, which carry a strong net negative charge, will move toward the anode.

An electrode for use according to the invention must conduct electricity efficiently, and preferably is inert, non-reactive and non-toxic under the conditions used. Specifically, an
10 electrode for internal use should not react with biological materials to any appreciable degree, *e.g.*, to avoid releasing metal ions from the electrode that could be harmful or poisonous, or to avoid oxidation that reduces electrode efficiency. A preferred material for electrodes of the invention is stainless steel, which is fairly non-reactive, reasonably efficient for conducting electricity, and inexpensive enough to be manufactured at a reasonable cost. More ideal
15 electrodes, particularly for internal use, are gold or platinum. However, such noble metal electrodes are very expensive. The cost of these materials can be reduced by plating them over other conductors. Other conductive metals include copper, silver or silver chloride, tin, nickel, lithium, aluminum, and iron, and amalgams thereof. However, certain materials, such as aluminum, should not be used internally. Electrodes can also be formed from zirconium,
20 iridium, titanium, and certain forms of carbon.

Some electrodes, such as silver and copper, have antibacterial activity, which could be desirable for internal administration to suppress infection.

The electrodes can be formed in any configuration appropriate for the target tissue, including, without limitation, straight wires, coiled wires (straight and coiled wire electrodes are
25 ideal for catheter applications), conductive surfaces (*e.g.*, of catheters or balloon catheters; see US Patent No. 5,704,908, incorporated herein by reference in its entirety), metal strips, needles (or probes), arrays of needles, surface electrodes, or combinations thereof. Contemplated electrode combinations include (1) a catheter electrode and a needle electrode; (2) a catheter electrode and a surface electrode; and (3) a needle electrode and a surface electrode. In a
30 specific embodiment, a needle electrode can be used with a syringe to deliver DNA. Such a needle electrode can have holes through the length of its shaft to permit delivery of the nucleic acid solution throughout its length. For delivery of a nucleic acid to a large organ, particularly a large muscle, two surface electrodes can be used. Surface electrodes are preferably used in combination with an electrolytic composition to ensure good contact and conductance, *e.g.*,
35 through the skin, as described above.

In a specific embodiment having one internal electrode and one external, the external electrode can have multiple "heads" placed around the internal one. Indeed, in general for any of the configurations set forth above, one of the electrodes can have multiple "heads."

5 The invention further contemplates arrays of electrodes; needles with holes along the shaft; needles with defined and calibrated conductive length (to provide constant and reproducible conductive area into a tissue, whatever the depth of the penetration of the needle into a tissue), with the upper and lower parts being electrically isolated; needles with isolated points, to prevent point to point electrical arcs into a tissue; and any kind of pouch / reservoir containing the product around one needle.

10 As set forth above for needle electrodes, plate electrodes can comprise isolated margins.

Generally, the electrodes are arranged so that the target tissue is directly between them. That way, the nucleic acids are subject to the maximum field strength. However, since the electric field flux is all around the electrode, it is possible to use the field generated peripherally between the electrodes as well as the field generated directly between the electrodes.

15

Modified Devices of the Prior Art

In a specific embodiment, an apparatus in which electrodes are positioned externally with respect to the patient (see, for example, US Patent Nos. 5,318,514 to Hofmann; 5,439,440 to Hofmann; 5,462,520 to Hofmann; 5,464,386 to Hofmann; 5,688,233 to Hofmann et al.; and
20 5,019,034 to Weaver et al.) is modified in accordance with the present invention, i.e., to supply an electric field under the defined conditions, to provide an improved apparatus of the invention.

The improved apparatus can be used non-invasively by applying the electrodes to the skin of the patient or invasively by applying the electrodes to the surface of an organ that has been exposed surgically.

25 Similarly, an electrotransfer system that utilizes implantable or insertable electrodes placed inside the patient to deliver an electric field to the area adjacent to the implanted/inserted electrode, particularly a catheter electrode (see, for example, US Patent Nos. 5,304,120 to Crandell et al.; 5,507,724 to Hofmann et al.; 5,501,662 to Hofmann; 5,702,359 to Hofmann et al.; and 5,273,525 to Hofmann) can be modified in accordance with the present invention to
30 yield an improved apparatus of the invention.

Naturally, an electrotransfer device that combines features of the above-mentioned systems, e.g., that utilize at least one internally placed electrode and at least one externally placed electrode to deliver the electric field to the desired tissue area (see, for example, US Patent No. 5,286,254 to Shapland et al.; 5,499,971 to Shapland et al.; 5,498,238 to Shapland et

al.; 5,282,785 to Shapland et al.; and 5,628,730 to Shapland et al) can be modified in accordance with the present invention to provide an improved apparatus of the invention.

Gene Therapy Using An Electrotransfer System

5 The process according to this invention is useful for gene therapy, that is, therapy in which the expression of a transferred gene, but also the modulation or blocking of a gene, makes it possible to ensure treatment of a particular pathology.

The tissue cells are preferably treated with a view to gene therapy making possible:

- the correction of dysfunctions of the cells themselves (for example, for treatment of diseases
10 related to genetic deficiencies like, for instance, mucoviscidosis or muscular dystrophy);
- the protection and/or regeneration of vascularization or innervation of the tissue, such as muscles, organs or bone, by trophic, neurotrophic, angiogenic factors, or by anti-inflammatory factors produced by the transgene;
- the transformation of muscle into an organ secreting products leading to a therapeutic effect,
15 such as the product of the gene itself (for example, thrombosis and hemostasis regulation factors, trophic factors, growth factors, hormones like insulin, erythropoietin, and leptin, etc.) or such as an active metabolite synthesized in the muscle by addition of the therapeutic gene e.g., to correct a genetic disease by secretion of a therapeutic product;
- delivery of anti-tumor genes such as tumor suppressors (retinoblastoma protein, p53, p71),
20 suicide genes (e.g., HSV-thymidine kinase), anti-angiogenesis genes (e.g., angiostatin, endostatin, amino terminal fragment of urokinase), cell cycle blockers, apoptosis genes (such as BAX) intracellular single chain antibodies, and immunostimulatory genes.
- a nucleic acid vaccine or immunostimulant gene.

25 The particular advantage of use of electrotransfer in gene therapy for a systemic problem by expression in the muscle resides in numerous factors:

- the remarkable stability of transgene expression, exceeding several months and therefore making possible the stable and sustained production of an intramuscular or secreted therapeutic protein,
- the ease of access to the muscular tissue, making possible direct, rapid and safe
30 administration in a nonvital organ,
- the large volume of the muscular mass, making possible multiple administration sites.
- the amply demonstrated secretory capacity of the muscle.

Added to these advantages is the safety contributed by local treatment associated with the use of local and targeted electric fields.

With the safety associated with the use of weak fields, this invention could be applied to the cardiac muscle for the treatment of heart diseases, *e.g.*, using cardiac pacing to ensure safe electrotransfer (see US Patent No. 5,634,899). It could also be applied to the treatment of restenosis by the expression of genes inhibiting smooth muscle cell proliferation like the GAX protein.

The combination of fields of low intensity and long duration of administration, applied notably to tissues *in vivo*, enhances the transfection of nucleic acids, without causing notable tissue deterioration. These results enhance the efficiency of DNA transfers in gene therapy employing nucleic acids.

Consequently, the advantages associated with the invention are the production of an agent at physiologic and/or therapeutic doses either in the tissues or in proximity thereto, or secreted systemically in the blood stream or lymph circulation. Furthermore, the invention makes possible, for the first time, fine modulation and control of the effective quantity of transgene expressed by the possibility of modulating the volume of the tissue to be transfected, for example, with multiple administration sites, or even the possibility of modulating the number, shape, surface and arrangement of the electrodes. An additional element of control arises out of the possibility of modulating the effectiveness of transfection by variation of the field intensity, number, duration and frequency of the pulses and, of course, according to the state of the art, the quantity and volume of administration of nucleic acids. A particular advantage of the present invention is the excellent dose-response curve achieved for DNA transfer, which none of the prior art methods have achieved. One can thus obtain a level of transfection appropriate to the level of intra-tissue production or secretion desired. The process makes possible, finally, extra safety in relation to the chemical or viral methods of *in vivo* gene transfer, in which reaching organs other than the target organ cannot be totally ruled out and controlled. In fact, the process according to the invention makes possible the control of localization of the tissues transfected (strictly linked to the volume of tissue subjected to local electric pulses) and therefore introduces the possibility of suppressing transgene expression by the total or partial ablation of the tissue, which is possible because certain tissues are not critical or can regenerate, or both, as in the case of muscle. That great flexibility of use makes it possible to optimize the process according to the animal species (human and veterinary applications), the subject's age and his or her physiological and/or pathological condition.

The process according to the invention further makes it possible, for the first time, to transfect large-sized nucleic acids, in contrast to viral methods, which are limited with respect to the size of a transgene by the size of the viral genome that can fit within the capsid. This

possibility is essential for the transfer of very large-sized genes, like that of dystrophin or genes with introns and/or large-sized regulator elements, which is necessary, for example, for a physiologically regulated production of hormones. That possibility is essential for the transfer of artificial yeast episomes or chromosomes or minichromosomes.

5 Another object of the invention is linking of the electric pulses of a voltage field to compositions containing nucleic acids formulated with a view to any administration, making it possible to access the tissue by topical, cutaneous, oral, vaginal, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal route, etc. The pharmaceutical compositions of the invention contain a pharmaceutically acceptable vehicle for
10 an injectable formulation, notably, for a direct injection into the desired organ, or for any other administration. It can involve, in particular, sterile isotonic solutions or dry compositions, notably lyophilized, which, with addition, as the case may be, of sterilized water of physiological saline, make possible the composition of injectable solutions. The nucleic acid doses used for injection as well as the number of administrations and volume of injections can
15 be adapted to different parameters and, notably, to the method of administration, the pathology involved, the gene to be expressed or even the duration of treatment sought.

Target Tissues

The present inventors have discovered that optimum conditions for gene transfer
20 according to the invention differ depending on the target tissue. For example, an electric field of 200 volts/cm has been found to greatly enhance gene transfer into muscle cells. Under these conditions, significant gene transfer proceeds into tumor cells as well (in specific experiments, a 3-fold enhancement of gene transfer was observed), but gene transfer into tumor cells is much more efficient in an electric field of 400 volts/cm (2 log increase in gene transfer efficiency). In
25 further experiments, an electric field strength of 500 volts/cm was optimum for gene transfer into tumor cells.

Thus, according to the present invention, a system or improved apparatus can be made for delivery of nucleic acids to muscle cells (and other large cells), and a system or improved apparatus having different electric field strength parameters can be developed for delivery of
30 genes to tumor cells (and other small cells).

For delivery of nucleic acids to muscle cells, a system or apparatus of the invention will generate a voltage gradient of between 1 and 400 volts/cm, preferably 4 to 400 volts/cm, more preferably 30 to 300 volts/cm. In specific embodiments, the voltage gradient is between 100 and 200 volts/cm. Particularly contemplated are systems or apparatus that provide a voltage

gradient that does not exceed 200 volts/cm.

For delivery of nucleic acids to tumor cells, a system or apparatus of the invention will generate a voltage gradient of between 1 and 600 volts/cm, preferably 100 to 600 volts/cm, more preferably 400 to 600 volts/cm. In specific embodiments, the voltage gradient is between 400
5 and 500 volts/cm, and preferably about 500 V/cm. Particularly contemplated are systems or apparatus that provide a voltage gradient that does not exceed 600 volts/cm.

Nucleic Acids

The nucleic acids can be of synthetic or biosynthetic origin, or extracted from viruses or
10 prokaryotic cells or eukaryotic cells originating from unicellular organisms (*e.g.*, yeasts) or multicellular organisms. They can be administered linked in whole or in part to components of the original organisms and/or system of synthesis.

The nucleic acid can be a deoxyribonucleic acid or a ribonucleic acid. It can involve sequences of natural or artificial origin and, notably, genomic DNA, cDNA, mRNA, tRNA and
15 rRNA, hybrid sequences or synthetic or semisynthetic sequences of oligonucleotides, whether modified or not. Those nucleic acids can be obtained by any method known to the expert and, notably, by cloning, by chemical synthesis or even by mixed methods, including chemical or enzymatic modification of sequences obtained by cloning. They can be modified chemically.

In particular, the nucleic acid can be a DNA or an RNA with sense or antisense or
20 catalytic property like a ribozyme. "Antisense" refers to a nucleic acid having a sequence complementary to a target sequence, *e.g.*, an mRNA sequence whose expression it is sought to block by hybridization on the target sequence. "Sense" refers to a nucleic acid having a sequence homologous or identical to a target sequence, *e.g.*, a sequence linked to a protein transcription factor and involved in the expression of a given gene. According to one preferred
25 embodiment, the nucleic acid contains a gene of interest and elements making possible the expression of said gene of interest. The nucleic acid fragment is advantageously in the form of a plasmid.

Deoxyribonucleic acids can be single or double-strand, like short oligonucleotides or longer sequences. They can carry genes, sequences regulating transcription or replication or
30 regions of linkage to other cell components, etc. Such genes can include marker genes, *i.e.*, genes that produce a detectable marker to study cell function, migration, or gene function; a therapeutic gene; a protective antigen or immunogen gene; and the like. According to the invention, "therapeutic gene" refers, notably, to any gene coding for an RNA or for a protein product having a therapeutic effect. The coded protein product can be a protein, a peptide, etc.

This protein product can be homologous with the target cell (that is, a product which is normally expressed in the target cell when it presents no pathology). In that case, the transgene expression makes it possible, for example, to overcome an inadequate expression in the cell or the expression of a protein inactive or weakly active by reason of a modification, or also makes
5 it possible to overexpress said protein. The therapeutic gene can also code for a mutant of a cellular protein, having an increased stability, a modified stability, etc. The protein product can likewise be heterologous to the target cell. In that case, an expressed protein can, for example, complete or introduce a deficient activity in the cell (treatment of myopathies or enzyme deficiencies), or make it possible to fight against a pathology, or stimulate an immune response,
10 for example, in the treatment of tumors. It can involve a suicide gene (thymidine kinase of herpes) for the treatment of cancers or restenosis.

The nucleic acid preferably includes also sequences making possible and/or favoring expression in the tissue of the therapeutic gene and/or gene coding for the antigenic peptide. It can involve sequences which are naturally responsible for the expression of the gene considered
15 when those sequences are capable of functioning in the transfected cell. It can also involve sequences of different origin (responsible for the expression of other proteins, or even synthetic ones). Notably, it can involve eukaryotic or viral gene promoter sequences. For example, it can involve promoter sequences originating from the genome of the cell it is desired to transfect. Among the eukaryotic promoters, one can use an inducer or repressor sequence to provide for
20 specific expression of the gene. Strong or weak, constitutive or inducible, promoters may be used. Ubiquitous (constitutive) promoters include HPRT, vimentin, α -actin, tubulin, etc. promoters. Tissue-specific promoters include (elongation factor-1- α , flt, flk) may be used. Inducible promoters include promoters responsive to hormones (such as steroid receptors, retinoic acid receptors, etc.), or promoters regulated by antibiotics (tetracycline, rapamycin, etc.) or other natural or synthetic molecules. It can likewise involve promoter sequences
25 originating from the genome of a virus. In that connection, one can, for example, mention the promoters of EIA, MLP, CMV, RSV genes, etc. Furthermore, these expression sequences can be modified by addition of sequences of activation, regulation, permitting conditional, transitory, or temporal expression, tissue specific expression, or general expression, etc.

30 In addition, the nucleic acid can also contain, particularly above the therapeutic gene, a signal sequence directing the therapeutic product synthesized into the secretory ducts of the target cell. This signal sequence can be the natural signal sequence of the therapeutic product, but it can also involve any other functional signal, or an artificial signal sequence. The nucleic acid can also contain a signal sequence directing the synthesized therapeutic product to a

particular cellular compartment, such as, for example, mitochondria for treatment of a mitochondrial genetic disease.

Therapeutic Genes and Gene Products

5 Among the therapeutic products according to the invention, one can particularly mention enzymes, blood proteins, hormones such as insulin or growth hormone, lymphokines: interleukins, interferons, tumor necrosis factors (TNF), etc. (French patent No. 92 03120), growth factors, *e.g.*, angiogenic factors such as VEGF or FGF.

10 For treatment of neuropathies, genes encoding neurotransmitters or their precursors or enzymes that synthesize neurotransmitters, trophic factors, particularly neurotrophic factors for the treatment of neurodegenerative diseases, damage to the nervous system caused by trauma or injury, or retinal degeneration, can be delivered with a system of the invention. For example, members of the family of neurotrophic factors include, but are not limited to, nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), NT4/5, NT6
15 (including allelic variants, and members of the same gene family). Other neurotrophins include members of the ciliary neurotrophic factor family, including ciliary neurotrophic factor (CNTF), axokine, leukemia inhibitory factor; other factors include IL-6 and related cytokines; cardiotrophin and its related genes; glial-derived neurotrophic factor (GDNF) and related genes; and members of the insulin-like growth factor (IGF) family, such as IGF-1, IGF-2; members of
20 the fibroblast growth factor family, such as FGF1 (acidic FGF), FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, etc.; members of the tumor growth factor family, such as TGF β ; HARP/pleiotrophin, or bone growth factors; hematopoietic factors, etc.

25 Other genes of interest code for muscle proteins of therapeutic benefit, both secreted and non-secreted, such as dystrophin or a minidystrophin (French patent No. 91 11947), or α -1-antitrypsin.

Other genes of interest code for factors involved in coagulation: factors VII, VIII, IX; suicide genes (thymidine kinase, cytosine deaminase); hemoglobin genes or other protein carriers.

30 In still another embodiment, genes corresponding to the proteins involved in lipid metabolism can be delivered, such as an apolipoprotein type chosen among the apolipoproteins A-I, A-II, A-IV, B, C-I, C-II, C-III, D, E, F, G, H, J and apo(a), and metabolic enzymes such as, for example, lipoprotein lipase, hepatic lipase, lecithin cholesterol acyltransferase, 7- α -cholesterol hydroxylase, phosphatidyl acid phosphatase, or even lipid transfer proteins like the transfer protein of cholesterol esters and the transfer protein of cholesterol esters and the transfer

protein of phospholipids, an HDL-binding protein or even a receptor chosen, for example, among the LDL receptors, chylomicron remnant receptors and scavenger receptors, etc. One can further add leptin for the treatment of obesity. One can also add the p53 anti-oncogene or other tumor suppressers like HSV-tk or even the GAX protein limiting cell proliferation in the smooth muscles (treatment of restenosis).

Other genes of interest include angiogenic factors, including vascular endothelial growth factors (VEGF, VEGF-2, VEGF-3, platelet growth factors), and angiostatin. On the other hand, delivery can be effected of genes encoding inhibitors of angiogenesis, particularly tumor angiogenesis, such as soluble receptors of angiogenic factors, specific inhibitors of the angiogenic factor receptors (Tie2, urokinase receptor, flt1, KDR), antibodies (including single chain Fv antibodies) against angiogenic factors (*e.g.*, anti-VEGF or anti-FGF), anti-integrin antibodies, endothelial tumor-specific toxins, polypeptide inhibitors of angiogenesis (amino terminal fragment of urokinase – ATF, angiostatin, endostatin, interferon- α or β , interleukin-12, platelet factor 4, TNF α , thrombospondin, platelet activating factor (PAI)-1, PAI2, TIMP1, prolactin fragment, etc.

Among the other proteins or peptides that can be secreted by a tissue or produced by that tissue, it is important to stress the antibodies, the variable fragments of single-chain antibodies (ScFv) or any other antibody fragment possessing recognition capacities for use in immunotherapy, *e.g.*, for the treatment of infectious diseases, tumors, and autoimmune diseases such as insular sclerosis (anti-idiotypic antibodies). Other proteins of interest are, without limiting them, soluble receptors like, for example, the CD4 soluble receptor or the TNF soluble receptor for anti-HIV therapy, soluble TNF receptor (particularly soluble TNF α receptor) for treatment of rheumatoid arthritis, and the acetylcholine soluble receptor for the treatment of myasthenia; substrate peptides or enzyme inhibitors, or even receptor agonist or antagonist peptides or adhesion proteins like, for example, for the treatment of asthma, thrombosis, restenosis, metastases, or inflammation (for example IL-4 to diminish Th1 cells responses, IL-10 and IL-13); and artificial, chimerical or truncated proteins.

Among the hormones of essential interest, one can mention insulin in the case of diabetes, growth hormone and calcitonin.

To enhance an antitumoral or antiinfective immune response, one may supply genes encoding immunostimulatory cytokines, including IL-2, IL-12, colony stimulating factors (GM-CSF, G-CSF, M-CSF), macrophage inflammatory factors (MIP1, MIP2), dendritic cell activating factors (flt3 ligand), etc.

Other genes of interest have been described by McKusick, V.A. Mendelian (Inheritance

in man, catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes. Eighth edition. Johns Hopkins University Press (1988)), and in Stanbury, J.B. et al. (The metabolic basis of inherited disease. Fifth edition, McGraw-Hill (1983)). The genes of interest cover the proteins involved in the metabolism of amino acids, lipids and other cell constituents.

5 One can thus mention the genes related to diseases of carbohydrate metabolism like, for example, fructose-1-phosphate aldolase, fructose-1,6-diphosphatase, glucose-6-phosphatase, lysosomal α -1,4-glucosidase, amylo-1,6-glucosidase, amylo-(1,4:1,6)-transglucosidase, muscular phosphorylase, muscular phosphofructokinase, phosphorylase-b-kinase, galactose-1-phosphate uridyl transferase, all of the enzymes of the pyruvic dehydrogenase complex, pyruvic
10 carboxylase, 2-oxoglutarate glyoxylase carboxylase, and D-glyceric dehydrogenase.

One can also mention:

- the genes related to diseases of amino acid metabolism like, for example, phenylalanine hydroxylase, dihydrobiopterine synthetase, tyrosine aminotransferase, tyrosinase, histidinase, fumarylaceto-acetase, glutathion synthetase, γ -glutamylcysteine synthetase,
15 ornithine-d-aminotransferase, carbamoylphosphate synthetase, ornithine carbamoyltransferase, argininosuccinate synthetase, argininosuccinate lyase, arginase, L-lysine dehydrogenase, L-lysine ketoglutarate reductase, valine transaminase, leucine isoleucine transaminase, branched-chain 2-keto-acid decarboxylase, isovaleryl-CoA dehydrogenase, acyl-CoA dehydrogenase, 3-hydroxy-3-methylglutaryl-CoA lyase, acetoacetyl-CoA 3-ketothiolase, propionyl-CoA carboxylase,
20 methylmalonyl-CoA mutase, ATP : cobalamine adenosyltransferase, dihydrofolate reductase, methylene tetrahydrofolate reductase, cystathionine β -synthetase, the sarcosine dehydrogenase complex, proteins belonging to the glycine cleavage system, β -alanine transaminase, serum carnosinase, and cerebral homocarnosinase.

- The genes related to diseases of fat and fatty acid metabolism like, for example,
25 lipoprotein lipase, apolipoprotein C-II, apolipoprotein E, other apolipoproteins, lecithin cholesterolacetyltransferase, LDL receptor, liver sterol hydroxylase, and "phytanic acid" α -hydroxylase.

- The genes related to lysosomal deficiencies like, for example, lysosomal α -L-iduronidase, lysosomal iduronate sulfatase, lysosomal heparan N-sulfatase, lysosomal N-acetyl- α -D-glucosaminidase, acetyl-CoA : lysosomal α -glucosamine N-acetyltransferase, lysosomal N-acetyl- α -D glucosamine 6-sulfatase, lysosomal galactosamine 6-sulfate sulfatase, lysosomal β -galactosidase, lysosomal arylsulfatase B, lysosomal β -glucuronidase, N-acetylglucosaminyl-phosphorotransferase, lysosomal α -D-mannosidase, lysosomal α -neuraminidase, lysosomal aspartylglucosaminidase, lysosomal α -L-fucosidase, lysosomal acid lipase, lysosomal acid

ceramidase, lysosomal sphingomyelinase, lysosomal glucocerebrosidase and lysosomal galactocerebrosidase, lysosomal galactosylceramidase, lysosomal arylsulfatase A, α -galactosidase A, lysosomal acid β -galactosidase, and a chain of lysosomal hexosaminidase A.

One can also mention, nonrestrictively, the genes related to diseases of steroid and lipid metabolism, the genes related to diseases of purine and pyrimidine metabolism, the genes related to diseases of porphyrine and heme metabolism, and the genes related to diseases of connective tissue, muscle and bone metabolism as well as the genes related to diseases of the blood and hematopoiesis, muscles (myopathy), nervous system (neurodegenerative diseases) or circulatory system (treatment of ischemia and stenosis, for example).

The numerous examples above and those that follow illustrate the potential scope of the area of application of this invention. The Examples, *infra*, demonstrate expression of each of the following factors.

Electrotransfer of NT3. Among the highly desirable genes for electrotransfer to muscle tissue is neurotrophin 3 (NT3). It has already been found that NT3 delivered intramuscularly in an adenovirus vector or in a non-viral vector increases the survival of *pmn* mice (Haase et al., Nature 3:429-436, 1997). This is a useful animal model for amyotrophic lateral sclerosis (ALS), commonly called "Lou Gehrig's Disease." Treatment of this insidious disease with NT3 gene therapy is expected to be greatly facilitated by delivering the NT3 by electrotransfer, which ensures adequate, reproducible expression of this trophic factor.

Electrotransfer of acidic FGF or VEGF. Another highly desirable gene for electrotransfer to muscle tissue is acidic FGF (aFGF; ECGF) or vascular endothelial growth factor (VEGF). Both of these have been found to be effective in treating arterial occlusive disease. Treatment of this disease with aFGF or VEGF electrotransfer-enhanced gene therapy is expected to further increase the efficiency of vascular growth. By applying the electric field in a paced manner, particularly through active cardiac pacing, therapy for cardiac artery occlusive disease will also be possible.

A therapeutic nucleic acid can also be a gene or an antisense sequence, whose expression in the target cell makes it possible to control the gene expression of cell mRNA transcription. Such sequences can, for example, be transcribed in the RNA target cell complementing cell mRNAs and thus block their protein translation, according to the method described in European patent No. 140,308. The therapeutic genes also include sequences coding for ribozymes, which are capable of selectively destroying target RNAs (European patent No. 321,201).

Immunogenic Genes and Vaccines

As indicated above, nucleic acid can also contain one or more genes coding for an immunogenic or an antigenic peptide, capable of generating an immune response in man or in the animal. In this particular embodiment, the invention therefore makes possible vaccines or immunotherapeutic treatments applied to man or to the animal, notably, against microorganisms, viruses or cancers. It can involve, notably, specific antigenic peptides of the Epstein-Barr virus, HIV virus, hepatitis B virus (European patent No. 185,573), pseudo-rage virus, "syncytia forming virus," other viruses or even specific antigens of tumors like MAGE proteins (European patent No. 259,212), or antigens capable of stimulating an antitumoral response, such as bacterial heat shock proteins.

Vectors

In the process according to the invention, the nucleic acid can be linked to any type of vectors or any combination of those vectors making it possible to enhance gene transfer, *e.g.*, without limitation, to vectors such as viruses, synthetic or biosynthetic agents (*e.g.*, lipid, polypeptide, glycoside or polymer), or even balls, propelled or not. The nucleic acids can also be injected into a tissue which has undergone a treatment aimed at enhancing gene transfer, *e.g.*, a treatment of a pharmacological nature in local or systemic application, or an enzyme, permeabilizing (use of surfactants), surgical, mechanical, thermal or physical treatment.

The examples which follow are intended to illustrate the invention in a nonlimitative manner.

EXAMPLES

EXAMPLE 1: STANDARD ELECTROPORATION CONDITIONS

Standard electroporation condition, *e.g.*, as employed in the US Patent Nos. 5,468,223, 5,304,120, 5,507,724, 5,273,525, 5,318,514, 5,439,440, 5,462,520, 5,464,386, 5,019,034, and 5,389,069, and international patent publications WO 97/07826, for example, which are discussed in greater detail, *supra*, were tested and found to provide for low efficiency, and even inhibition, of nucleic acid (plasmid DNA) transfer in striated muscle.

Materials and Methods

In this example the following products were used:

DNA pXL2774 (PCT/FR patent 96/01414) is a plasmid DNA containing the reporter gene of luciferase. The other products are available at suppliers' on the market: ketamine,

xylazine, physiological saline (NaCl 0.9%).

An oscilloscope and a commercial electric pulse generator (rectangular or square) (Electropulsator PS 15, Jouan, France) were used). The electrodes used are flat stainless steel electrodes spaced 5.3 mm apart.

5 The experiment was conducted in the C57 B1/6 mouse. The mice, coming from different cages, were distributed at random before the experiment ("randomization").

 The mice were anesthetized with a ketamine and xylazine mixture. The plasmid solution (30 mg/ml of a solution with 500 mg/ml of NaCl 0.9%) was injected longitudinally through the skin into the cranial tibial muscle of the left and right paws by means of a
10 Hamilton's syringe. The two electrodes were coated with a conductive gel and the paw injected was placed between the electrodes in contact with the latter.

 The electric pulses were applied perpendicular to the axis of the muscle by means of a square pulse generator one minute after the injection. An oscilloscope made it possible to check the intensity in volts (the values indicated in the examples represent maximum values), the
15 duration in milliseconds and the frequency in hertz of the pulses delivered, which was 1 Hz. Eight consecutive pulses were delivered.

 For the evaluation of transfection of the muscle, the mice were euthanized seven days after administration of the plasmid. The cranial tibial muscles of the left and right paws were then removed, weighed, placed in the lysis buffer and ground. The suspension obtained was
20 centrifuged in order to obtain a clear supernatant. Measurement of luciferase activity was carried out on 10 ml of supernatant by means of a commercial luminometer, in which the substrate was added automatically to the solution. The intensity of the luminous reaction is given in RLU (Relative Luminescence Units) for a muscle experiencing the total volume of suspension. Each experimental condition was tested on 10 points: 5 animals injected bilaterally.
25 The statistical comparison was made by means of nonparametric tests.

Results and Discussion

Two figures, the scale of which is linear or logarithmic, illustrate the results.

30 In this first experiment the effects of an electric field of 800 to 1,200 volts/cm, which were conditions used for the electroporation of tumors, were tested (Mir et al., Eur. J. Cancer 27, 68, 1991; US Patent 5,468,223).

 It was observed, according to Figure 1, that, in relation to the control group, in which DNA was injected without electric pulse:

- with 8 pulses of 1,200 volts/cm and a duration of 0.1 msec, the mean value of luciferase

activity was much lower;

- with pulses of 1,200 volts/cm and 1 msec., three animals died and the mean value of luciferase activity was much lower;
 - with pulses of 800 volts/cm and 1 msec, the mean value of luciferase activity was also
- 5 significantly reduced.

Most of the muscles having undergone the action of the electric field were visibly altered (friable and of whitish appearance).

EXAMPLE 2: ELECTROTRANSFER OF NUCLEIC ACIDS

10 This experiment was carried out with C57 B1/6 mice. Apart from the electric field intensity of the pulses and their duration, the performance conditions were those of Example 1.

The results are shown on Figure 2. The result of Example 1 was reproduced, that is, the inhibiting effect of a series of 8 pulses at 800 volts/cm with duration of 1 msec on the luciferase activity detected in the muscle. With a field of 600 volts/cm, the same inhibition and the same

15 alteration of the muscular tissue was observed. However, shorter pulse widths at this voltage are likely to avoid tissue damage while enhancing DNA transfer. On the other hand, quite remarkably and surprisingly, the diminution of voltage made it possible to no longer visibly alter the muscles and, furthermore, at 400 and 200 volts/cm the level of transfection of the muscles was higher on the average than that obtained on muscles not subjected to a field. It is

20 to be noted that, in relation to the control group (not subjected to an electric field), the dispersion of luciferase activity values was significantly reduced at 200 volts/cm (SEM = 20.59% of the mean value, compared to 43.32% in the absence of the electric field (Figure 2A)).

As one of ordinary skill in the art can readily ascertain, these data confirm that a prior art apparatus for electrotransfer can be modified in accordance with the discoveries of the

25 present invention to yield a system or apparatus of the invention. Although the modification is simple, the results produced by the modification are totally unexpected. A system or apparatus of the invention produces an unexpected enhancement in both the efficiency and reproducibility of nucleic acid transfer, as demonstrated with the plasmid DNA transfer experiments reported in this example and in the following examples.

EXAMPLE 3: ELECTROTRANSFER ENHANCES TRANSGENE EXPRESSION

This experiment was carried out with C57 B1/6 mice. Apart from the electric field intensity of the pulses and their duration and the fact that the pulses were delivered 25 seconds after the injection of DNA, the performance conditions were those of the foregoing examples.

5 The results are shown on Figure 3. The mean value of the luciferase transgene expressed was markedly increased with an pulse duration of 20 msec at 100 volts/cm, starting from an pulse duration of 5 msec at 200 volts/cm.

 This experiment also clearly shows that the mean value of luciferase activity obtained by electrotransfection of DNA in the muscle is a function of the duration of the electric pulses, 10 when voltages of 200 and 100 volts/cm are used. It is also to be observed that the dispersion of values is notably reduced for the groups of muscles electrotransfected (Figure 3A). In the absence of electric pulses (control), the SEM represents 77.43% of the mean value, while the relative SEM is reduced to 14% under electric field conditions of 200 volts/cm with pulse times of 5 msec, 41.27% under electric field conditions of 200 volts/cm with pulse times of 20 msec, 15 and between 30% and 48% for electrotransfer at 100 volts/cm.

 In the best condition of this experiment, the transgene expression was enhanced by a factor of 89.7 in relation to the control injected in the absence of electric pulses.

EXAMPLE 4: 200-FOLD INCREASE IN EXPRESSION

20 This experiment was carried out in DBA 2 mice with electric pulses of 200 volts/cm of variable duration. The other conditions of this experiment are the same as in Example 3.

 This example confirms that at 200 volts/cm the transfection of luciferase activity increased when the pulse duration was increased from 5 msec to longer duration (Figures 4 and 5). A reduction of the interindividual variability indicated by the SEM in relation to the control 25 not electrotransfected was observed. The relative value of the SEM is equal to 35% for the control and 25, 22, 16, 18, and 26% for series of pulses of 1, 5, 10, 15, 20 and 24 msec respectively. Under the optimal conditions used in this experiment, the transgene expression was enhanced by a factor of 205 in relation to the control injected in the absence of electric pulses. These results confirm that electrotransfer under the conditions described in these 30 examples greatly improves both efficacy and reproducibility.

EXAMPLE 5: QUANTITATION OF THE EFFECTIVENESS OF ELECTROTRANSFER

 Figure 5 exemplifies the importance of the parameter corresponding to the product "number of pulses x field intensity x duration of each pulse." That parameter corresponds, in 35 fact, to the integral dependent on time of the function which describes the variation of the

electric field.

The data in Figure 5 were obtained from the results obtained in the course of Experiments 2, 3 and 5. Electric field intensities of 200 V/cm and 100 V/cm, or in the absence of electric fields, were evaluated. The data show that the effectiveness of transfection increases as a function of the product of the total duration of exposure to the electric field by the field intensity. Enhancement of nucleic acid transfer is obtained with a value exceeding 1 kVxmsec/cm of the product "electric field x total duration of pulses." According to a preferred embodiment, a stimulation is obtained for a value exceeding or equal to 5 kVxmsec/cm of the product "electric field x total duration of pulses."

EXAMPLE 6: WIDE APPLICABILITY OF ELECTROTRANSFER

Further experiments have been conducted that confirm the results of Examples 2-5, *supra*. Indeed, important, and reproducible enhancement of gene transfer has been observed in mouse, rat, and rabbit by using electric pulses of low voltage. The conditions used have previously been described as ineffective in other investigated cell types such as tumor, skin, or hepatic cells.

The following parameters have been varied and studied in this Example:

- *Characteristics of the electric field*: Voltage/ cm, number, frequency, duration of pulses, defining the best conditions on mice tibialis muscle as 200 V/ cm, 8 pulses of 20 ms at 1 to 2 Hz.
- *Shape / type of the electrodes*: most of the experiments were performed with non invasive plate electrodes; the feasibility of needle electrodes was demonstrated in a rabbit experiment.
- *Amount of DNA* (using best transfer conditions on mice tibialis muscle).
- *Different batches of DNA*.
- *Different reporter genes* : luciferase, LacZ (mice), FGF (rats).
- *Different animal species* : mice, rats, rabbits.
- *Different sites of injection* : tibialis, gastrocnemius, quadriceps on mice; tibialis on rats; tibialis, quadriceps and triceps on rabbits.
- *Distance* of injection site vs pulse site.
- *Timing* of injection vs application of electric pulses.
- *Different experimenters* performing injections and application of pulses.

The results observed were as follows:

- *A significant enhancement of gene transfer over naked DNA injection alone*: from 5-10

to 100-fold or even higher in mice muscles, 100- to 250-fold enhancement in rats, 100- to 50,000-fold in rabbits, depending of the level of the control.

- *A significant reduction of the inter-animal dispersion/ variability of the results.* Electric pulses have to be administered after DNA injection and at the same site. The DNA vector
5 can be administered up to 30 min before the pulse without noticeably decreasing the response. This allows multiple DNA injections at nearby sites followed by one single pulse.
- Experiments performed in mice with LacZ and followed by histological analysis confirmed that the procedure resulted in about 10-fold more muscle fibers expressing the gene. Data in rats with FGF also indicated a significant increase of the number of expressing cells.
- 10 • An experiment using secreted alkaline phosphatase, a secreted reporter protein gene, indicated also a two log enhancement factor for the secreted protein dosed in the serum.
- *Inverse dependence of transfection efficiency on size.* Smaller plasmids are more efficiently transferred; however, the electrotransfer device dramatically increases the efficiency of DNA transfer regardless of the size of the DNA, thus overcoming a significant
15 obstacle to transfection with YACs, cosmids, or artificial chromosomes.
- *Independence of the promoter and of protein processing.* Electrotransfer efficiency does not depend in any way on the transgene promoter, which permits another level of control of gene expression. In addition, the presence of secretory or other regulatory/processing sequences in the gene product has no effect on electrotransfer efficiency.

20 These experiments have shown that electrotransfer is not without some side effects, although the effects are minimal compared to electroporation conditions. Local inflammatory reaction and regeneration process were documented seven days after the pulse. This inflammation was mild and reversible. The electric pulses induce a general contraction in mice. In the rat, this effect is much decreased and localized in the hindlimb. In the rabbit, preliminary
25 experiments indicate that the contraction is restricted to the muscular group submitted to the pulse. There was no apparent pain reaction (no cry) by the anesthetized rats or rabbits. During the recovery from the anesthesia, none of the animal showed any apparent pain in the treated limbs.

30 These experiments demonstrate the superiority of electrotransfer devices used under the conditions described in this application, in terms of the enhancement of nucleic acid transfer, the reduction in intra-experimental variability, and the reduction or elimination of adverse side effects. These results are presented in greater detail in the examples below.

EXAMPLE 7: TRANSFECTION AS A FUNCTION OF PULSE DURATION

This example demonstrates the effect of increasing the pulse duration on transfection efficiency under electrotransfer conditions.

The experimental conditions were the same as those of Example 1 with C57Bl/6 mice, except that a Gentronics/BTX T820 pulse generator (BTX, a division of Genetronics, San Diego, California) was used. The BTX pulse generator enabled application of square pulses of durations up to 100 ms. Plasmid pXL2774 (WO 97/10343) was injected (15 µg). It is noted in Table 1 that at a constant electric field strength of 200 V/cm, increasing the duration (T) of the pulses improves the efficiency of the transfection.

These data establish optimized parameters for an electrotransfer device for delivery of nucleic acids to muscle. Such a device preferably provides a pulse of 20 msec or greater, with at least 4, and more preferably 8, pulses.

Table 1: Electrotransfer efficiency dependence on the pulse duration.

Pulse duration (msec)	0	1	5	10	20	30	40	50	60	80
Trial A 8 pulses	11 ± 5	39 ± 6	211 ± 26	288 ± 46	1158 ± 238	1487 ± 421	2386 ± 278			
Trial B 4 pulses	11 ± 5	26,8 ± 6	123 ± 17	246 ± 32	575 ± 88	704 ± 130		3440 ± 1077		
Trial C 4 pulses	15 ± 8						2885 ± 644		2626 ± 441	1258 ± 309

Median value of luciferase activity in millions of RLU per muscle +/- SEM, N=10. Electrotransfer conditions: 200 V/cm field strength, 1 Hz frequency.

These data show that a device for electrotransfer under the optimized field strength described in this application can further enhance the efficiency of DNA transfer by increasing the duration of the pulse. For example, increasing the duration to at least 40 msec with a series of 8 pulses, or 50 msec for a series of 4 pulses, significantly enhanced transfection efficiency at 200 V/cm. Similar optimizations of the device can be effected for other field strength.

EXAMPLE 8: TRANSFECTION AS A FUNCTION OF THE NUMBER OF PULSES

This example demonstrates the effect of increasing the number of pulses on the efficiency of transfection under electrotransfer conditions.

The experimental conditions were the same as described in Example 1, using C57Bl/6 mice. Table 2 shows that at 200 V/cm with a pulse duration of 20 ms, the efficiency of the transfection was clearly improved compared to the control group (no electric field applied),

starting from a single pulse, then continues to increase when the number of pulses is increased to 2, 4, 6, 8, 12, and 16, with the optimum between 8 and 16 pulses. Also to be noted is a reduction in the variance (S.E.M.) for all the electrotransfected groups compared to the control (0 pulses).

Table 2: Transfection efficiency versus number of pulses

No. of pulses	0	1	2	4	6	8	12	16
Mean ($\times 10^{-7}$)	69.57	147.91	281.02	438.62	678.44	818.73	929.20	889.75
S.E.M.	80.09	17.76	16.38	11.44	19.05	8.87	18.20	15.44

Average luciferase expression in millions of RLU per muscle, \pm S.E.M.; N=10 for each group; 200 V/cm field intensity; 1 Hz frequency.

These data show that an electrotransfer device enhances the efficiency of DNA transfer with more pulses. Under the field strength tested (200 V/cm), a device optimally provides 4 or more, and better yet, 8 or more, pulses. By modifying the number of pulses, an electrotransfer device can modulate the efficiency of nucleic acid transfer, and thus adjust the level of expression.

EXAMPLE 9: TRANSFECTION AS A FUNCTION OF FREQUENCY

This Example shows that the increasing the frequency of the pulses increase the efficiency of transfection. In clinical use, an electrotransfer device that applies the pulses at greater frequency enhances the patient's comfort, by reducing the overall length of time the electric field is applied. Thus, both efficiency and patient comfort are improved by increasing the frequency.

The experimental conditions were the same as described in Example 1 using C57Bl/6 mice. Plasmid pXL2774 (15 μ g) was injected. Frequency was varied from 0.1 to 4 Hz, with 8 or 4 pulses at a field strength of 200 V/cm and a duration of 20 ms. The results are shown in Table 3.

Table 3: Transfection efficiency versus frequency (Hz)

Frequency (Hertz)	0	0.1	0.2	0.5	1	2	3	4
Trial A 8 pulses	5 ± 2	54 ± 13	95 ± 16	405 ± 60	996 ± 156	1528 ± 257		
Trial B 4 pulses		114 ± 14	163 ± 24	175 ± 26	337 ± 53	587 ± 90		
Trial C 8 pulses	21 ± 14				1294 ± 189	2141 ± 387	3634 ± 868	2819 ± 493
Trial D 4 pulses					1451 ± 228	1572 ± 320	1222 ± 126	2474 ± 646

Average luciferase activity in millions of RLU per muscle \pm S.E.M.; N=10 for each group; electric field intensity 200 V/cm; pulse length 20 msec.

The results demonstrate that at higher frequency (greater than 1 Hz, and preferably greater than 2 Hz), under the electrotransfer conditions tested, transfection efficiency is increased.

EXAMPLE 10: ELECTROTRANSFECTION WITH AN EXPONENTIALLY DECREASING TIME-VARYING ELECTRIC FIELD.

This example shows the effect of application of an electric field that decreases exponentially on the efficiency of nucleic acid transfer *in vivo*.

C57Bl/6 mice were used in this experiment. Plasmid pXL3031 (Figure 12), derived from plasmid pXL2774 by introducing modified *Photinus pyralis* luciferase (pGL3; Genbank accession no. CVU47295) under control of the cytomegalovirus immediate early (CMV-IE) promoter (Genbank accession no. HS5IEE) and a polyadenylation signal from SV40 virus (Genbank accession no. SV4CG), was used in this experiment. Ten μ g of DNA were injected.

The commercial electropulser (Equibio electropulsater, model Easyject^T Plus, Kent, UK) used here was configured to deliver exponentially decreasing time-varying electric field pulses. The recorded voltage applied is the voltage at the peak of the exponential. The second adjustable parameter is the capacitance (in μ F), which controls the quantity of energy delivered.

Table 4 shows that, when an exponentially decreasing field pulse is applied, it is possible to obtain a very clear increase in the expression of the transgene compared to the case when no field is applied. This result is obtained at different voltages and for different energies corresponding to different time constants of the exponential, which may be modulated by the adjustable capacitance of the instrument. The parameters established in this example can be applied to an electrotransfer device.

Table 4: Transfection efficiency (luciferase activity relative to control) with exponentially decreasing, time-varying field

	Capa μ F 150	Capa μ F 300	Capa μ F 450	Capa μ F 600	Capa μ F 1200	Capa μ F 2400	Capa μ F 3000
40 V/cm						1,23	11
100 V/cm				16,5	2,8	6,5	23,9
150 V/cm				1,8	3,5	6,1	
200 V/cm		5,1		15,8	18,8	121,5	189,7
300 V/cm	32,1	90,5	48,7	760,4	56,2		
400 V/cm		795					
600 V/cm	62						
800 V/cm	3,1	1,1					

5

Increase in the level of expression of luciferase relative to control levels, which were established by injection of plasmid pXL3031 without electrotransfer conditions. The average value for the increase in the level of expression is represented; N=4 to 6 mice per test.

10

By way of comparison, the increase in luciferase activity using a square wave pulse at 200 V/cm, 8 pulses of 20 msec each, at a frequency of 1 Hz was 44.

These data show that an electrotransfer device that applies an exponentially decreasing electric field with time can increase the expression at lower electric field strength, with greater capacitance (e.g., 200 V/cm, capacitance of 3000 μ Farad).

15

EXAMPLE 11: COMBINATION OF A SHORT HIGH-VOLTAGE PULSE AND SEVERAL LONG LOW-VOLTAGE PULSES

This example shows that the electric field delivered may be a combination of at least one strong field between 500 and 800 V/cm for a short period, for example 50 or 100 μ s, and at least one weak field (< 100 V/cm) for a longer time, for example, more than 1 ms up to 90 ms in this experiment. The weak electric field values here are 80 V/cm applied in 4 pulses at 1 Hz with a duration of 90 ms each. For this experiment, two commercial electropulsaters were used (Jouan and Gentronix). The delivery of the electrical voltage by one and then the other instrument to the electrode plates occurs in less than one second by modifying the operation configuration manually.

The luciferase-encoding plasmid used here was pXL3031 and the quantity injected was 3 μ g. The values for the electric field strength were varied, as reported in Table 5. Otherwise, the experimental conditions were the same as described in Example 1.

25

Table 5 summarizes the experiments. These data indicate that, compared to the control

group (no electric field applied), one short high-voltage pulse or 4 long low-voltage pulses, or the application of weak electric field pulses before the high field pulse, did little to improve transfection efficiency. In contrast, in the experiments, the combination of a short high voltage pulse followed by 4 pulses of 80 V/cm of 90 ms duration at 1 Hz very clearly increased transfection compared to the control group. From these data, it appears that a preferred electrotransfer device would supply a series of shorter pulses at higher electric field strength

Table 5: Combination of Pulses of Varying Strength and Time

Electric Field Conditions	Experiment 1 (3 μ g pXL3031)	Experiment 2 (3 μ g pXL3031)
Control (no electric field applied)	320 \pm 126	75 \pm 27
A : 500 V/cm , 1 x 0.1 msec	-	169 \pm 63
B : 800 V/cm , 1 x 0.1 msec	416 \pm 143	272 \pm 84
C : 80 V/cm , 4 x 90 msec, 1 Hz	1282 \pm 203	362,21 \pm 85,17
Conditions: A, followed by C	-	1479 \pm 276
Conditions: B, followed by C	3991 \pm 418	1426 \pm 209
Conditions: C, followed by B	-	347 \pm 66

10

As shown in Example 1, *supra*, application of 8 pulses of 600, 800, or 1200 V/cm for 1 msec at a frequency of 1 Hz caused muscle lesions and inhibited transfection. The results obtained in this Example show that, under the specified conditions, it is possible to use an high voltage electric field without causing lesions. Indeed, macroscopic examination of the muscle did not evidence any visible atleration. Use of a high voltage field for a short time, followed by weak fields for longer time periods, provides an alternative means for modulating the efficiency of DNA transfer.

15

EXAMPLE 12: KINETICS OF LUCIFERASE EXPRESSION IN MUSCLE

20

Use of an electrotransfer device of the application permits transfection and stable expression of a nucleic acid at a high level for at least four months.

C67Bl/6 mice were used in this experiment. The mice were injected intramuscularly with plasmid pXL2774 (15 μ g). Injection of the DNA was followed by application of an electric field under the following conditions: 200 V/cm; 8 pulses of 20 msec duration; 1 Hz frequency. Other

conditions were as described in Example 1. Luciferase activity was determined for groups of 10 mice sacrificed at different times after injection of the DNA. Control mice were not exposed to the electric field.

The data in Figure 6 show that the expression of luciferase is detectable from the third hour after the injection of the plasmid and increases until the third day (D3). Luciferase expression begins to decline from day 35. For the groups treated by the electric field, it is noteworthy that the transfection was very clearly increased regardless of the time of measurement of the level of expression. On day 121 (D121) the difference between the control and the treated groups was even more pronounced since expression of the transfected DNA was retained after electrotransfer whereas expression in the control muscle declined.

More remarkably, the level of expression of the transgene was stable to D121. This result is especially advantageous from the perspective of long term clinical treatment with therapeutic genes.

15 EXAMPLE 13: HISTOLOGY OF ELECTROTRANSFECTED MUSCLE

Histological analysis over the course of the kinetic experiment verified the absence of a critical inflammatory response. Moderate inflammation, indicated by the presence of macrophages and lymphocytes, was observed. This inflammatory reaction was greatly reduced by D121, while the level of transgene expression remained stable and high as shown in Figure 6.

20 Histological analysis was confirmed under these conditions, except that the plasmid pXL3004 (Figure 13) was used. This plasmid is a pCOR plasmid (pXL2774; see WO 97/10343) encoding β -galactosidase. The pXL2774 plasmid was modified by introduction of the *lacZ* gene modified with a nuclear localization signal sequence (see Mouvel *et al.*, 1994, *Virology* 204:180-189) under control of the CMV promoter obtained from plasmid pCDNA3 (Invitrogen, 25 Netherlands), with the SV40 polyadenylation signal (Genbank accession no. SV4CG). The animals were sacrificed seven days after administration of the plasmid. Histologic analysis allowed detection of β -galactosidase transfected cells (Xgal histochemistry) and the inflammatory foci by alumized carmine staining and the characterization of the muscle tissue condition by hematein-eosin staining. Control mice were not exposed to the electric field.

30 The differences between the electroporomeabilized and non-electroporomeabilized muscles were shown by:

- The number of myofibrils expressing β -galactosidase was 9 times greater myofibrils in the electrotransfected muscles (average of 76, N=6 mice) relative to controls (average of 8.5, N=8 mice). Most of these muscular fibers are quiescent with nuclei located peripherally. Very rare

centronuclear myofibrils (in regeneration) express β -galactosidase.

- The expression area of β -galactosidase was 2 times larger in the electroporabilized muscles (4 mm) compared to controls, with an expression gradient which decreases from the injection site.
- 5 • In this study, it is noted that the electroporabilized muscles present a reversible number of infiltrates (macrophages and lymphocytes), numerous muscular fibers in regeneration with a nuclear centralization, and numerous necrotic fibers filled with phagocytic macrophages. The inflammation, necrosis, and regeneration zone corresponds to the zone around the transfected myofibrils. This response lasted up to two weeks, and reversed itself. The non-transfected
10 part of the muscle remains in good condition.
- In the non-electroporabilized muscles, a few necrotic myofibrils and others in regeneration are localized around the injection site with a few inflammatory foci.

In short, these data show that while electrotransfer conditions result in observable inflammation, the inflammation is not significant, particularly in view of the dramatic
15 enhancement of nucleic acid transfer efficiency. Furthermore, the data from the kinetic study demonstrate that the inflammation reverses itself, even when transgene expression remains steady at a high level.

EXAMPLE 14: ROLE OF TIME OF INJECTION OF THE PLASMID RELATIVE TO THE
20 TIME OF APPLICATION OF THE ELECTRICAL FIELD

This Example demonstrates that the nucleic acid can be injected into tissue (in this case, muscle) at least 30 minutes, and even as long as one hour, prior to application of the electric field.

C57Bl/6 mice were injected intramuscularly with plasmid pXL2774 (15 or 1.5 μ g). The
25 DNA was injected up to 120 minutes before or 60 seconds after the electric field was applied. The time before or after injection is reported in Table 6. The electric field conditions used were: 200 V/cm; 8 pulses of 20 msec duration; 1 Hz frequency. Control mice received an injection of the plasmid but were not exposed to the electric field. Other experimental conditions were the same as those of Example 1.

30 The data are reported in Table 6. Injection of the DNA up to one hour prior to application of the electric field resulted in achievement of increased transfection efficiency, as detected by luciferase expression. The same trend observed with injection of 15 μ g of plasmid per muscle was also observed with injection of a dose 10 times lower, *i.e.*, 1.5 μ g of DNA. No

enhancement of DNA transfection efficiency was observed when the plasmid was injected after application of the electric field.

5 Table 6: Electrotransfer Efficiency of Plasmid Injected Before and After Application of the Electric Field

6A : Injection of DNA in the absence of electric field (control)

Time of injection relative to application of the electric field	<u>Exp 1</u>	<u>Exp 2</u>	<u>Exp 3</u>	<u>Exp 4</u>	<u>Exp 5</u>
	pXL2774 (15 µg)	pXL2774 15 µg)	pXL2774 (1,5 µg)	pXL2774 (15 µg)	pXL2774 (1,5 µg)
0	7 ± 4	8 ± 6	0.4 ± 0.2	22 ± 15	1 ± 1

6B : Injection of DNA prior to application of the electric field

<u>Time</u>	<u>Exp 1</u>	<u>Exp 2</u>	<u>Exp 3</u>	<u>Exp 4</u>	<u>Exp 5</u>
- 120 min				20 ± 5	2 ± 1
- 60 min				106 ± 22	10 ± 3
- 30 min	303 ± 36	237 ± 61	7 ± 3	184 ± 22	15 ± 4
- 5 min	410 ± 7				
- 60 sec	253 ± 51				
- 20 sec	492 ± 122	201 ± 43	9 ± 3	123 ± 23	12 ± 2

6C: Injection of DNA after application of the electric field

<u>Time</u>	<u>Exp 1</u>	<u>Exp 2</u>	<u>Exp 3</u>	<u>Exp 4</u>	<u>Exp 5</u>
+ 10 sec				7 ± 7	
+ 20 sec	11 ± 6	0.4 ± 0.1			
+ 60 sec	8 ± 7			17 ± 15	

In contrast to the results observed here, in which injection of the plasmid DNA up to one hour prior to application of the electric field provided for high level expression of the plasmid, *in vitro* various authors have observed that for electroporation, it is necessary that the plasmid be present at the time of the application of the electric field.

EXAMPLE 15: DOSE-RESPONSE WITH ELECTROTRANSFER

Statistical analysis presented in this Example permits comparison of the dose-response under electrotransfer conditions. This study confirmed that use of an electrotransfer device
5 greatly reduced variability of the level of expression of the plasmid.

Five-week-old C57BI16 mice were injected intramuscularly bilaterally in the tibial cranial muscles with doses varying from 0.25 to 32 μ g of DNA (plasmid pCOR-pXL3031) bearing the transgene luciferase for cytoplasmic expression, under the promoter CMVh at a rate of 10 mice per dose of DNA. The dose of DNA varied from 0.25 to 32 μ g. Immediately after the injection,
10 one of the two legs was exposed to a field of 250 V/cm, with 4 pulses of 20 ms at a frequency of 1 Hz. The animals were sacrificed 5 days after the treatment and the expression of the transgene was studied in the tissue extract of each muscle according to the protocol described in Example 1. Under these electrotransfer conditions, macroscopic observation of the muscles showed only two traces of slight thermal damage to the tissue out of 150 muscles subjected to the treatment.

15 Comparison of the change in variances as a function of that of the means for each series of mice (n=10) clearly shows that the distribution of the expression of the transgene is log-normal. The graphic analysis of the results (Figure 7) confirmed by calculation shows that the effect varies linearly with the logarithm of the dose of DNA injected.

With a Cochran test, it is possible to demonstrate that there is homogeneity of the
20 variances for each regression (with and without electrotransfer), a fact which enables using the residual variances to perform all the calculations. Variance from linearity was not significant to 5% confidence under electrotransfer conditions. In contrast, the variance from linearity was highly significant ($p < 0.01$), indicating significant heterogeneity of DNA transfection efficiency under standard conditions (non-electrotransfer conditions). The data show that residual variance
25 is 5 times greater in the case where there was no electrotransfer compared to that in which there was electrotransfer.

Considering the values estimated for the residual variances, it would be necessary to use 5 times as many animals to obtain the same power in a transfection efficiency comparison test under non-electrotransfer conditions compared to electrotransfer. This analysis translates into a clear
30 advantage of utilizing an electrotransfer device. To demonstrate a variation of two, five, or ten times the expression of the transgene with 95% confidence, it would be necessary to inject some 33, 8, or 5 animals under electrotransfer conditions, as compared to 165, 40, or 25 animals under non-electrotransfer conditions. A table summarizing this type of calculation is shown below in Table 7.

Table 7: Calculation of the number of animals for statistically significant plasmid expression with an electrotransfer device.

Efficiency ratio	P = 95%	P = 90%	P = 85%	P = 75%
2	33	28	24	19
5	8	7	6	6
10	5	5	4	4

5

These data show that the electrotransfer technique not only greatly increases the efficiency of transfection, it significantly reduces variability of responses. This method, and devices for implementing it, permits rigorous analytical studies of tissue transfection, as well as reproducible delivery of therapeutic genes within the window of therapeutic treatment.

10

A comparison test of the slopes obtained for each regression is not meaningful. It is thus possible to consider at a risk of 5% that there is parallelism of the two regressions. The calculation of relative power shows that to obtain the same effect, about 250 times more DNA must be injected per muscle under standard conditions compared to use of an electrotransfer device (243 ± 85 , with a confidence interval of 95%). This result may be translated as an increase approximately 500 times of the expression of the transgene for the same dose of DNA injected with electrotransfer compared to standard DNA injection.

15

This example shows that with two luciferase-encoding plasmids, it is possible to establish a significant linear correlation between the plasmid dose injected and electrotransfection. This correlation is much less significant without electrotransfer. Statistical analysis also demonstrates a significant reduction in variance for the electrotransfected groups. It is thus possible with the electrotransfer device of the invention to effectively and predictably modulate the level of expression of the transgene by varying the quantity of plasmid injected.

20

EXAMPLE 16: ELECTROTRANSFER WITH DIFFERENT ELECTRODES

25

This Example compares the effect of electrotransfer devices equipped with one of two types of electrodes, flat plate electrodes and needle electrodes, on the efficiency of nucleic acid transfer. In addition, needle electrodes were tested in different orientations of implantation.

30

Plasmid pXL2774 (150 μ g) was injected in the tricep muscle of the rat. The plate electrodes were placed as described in Example 1 at an inter-electrode distance of 1.2 cm. For the needle electrodes, the inter-electrode distance was 0.9 cm. The needle electrodes were inserted for an equal length in the muscle tissue, either parallel to or perpendicular to the axis of the muscle

fibers around the injection site. Regardless of the type of electrodes or their orientation, the electric field conditions were as follows: intensity of 200 V/cm; 8 pulses of 20 msec; frequency of 2 Hz.

The results of this experiment are shown in Figure 8. The data show that
5 electrotransfection was comparable regardless of the mode of application of the electric field. Similar levels of transfection were obtained with the needle electrodes and plate electrodes. In addition, electrotransfer efficiency appeared to be independent of the orientation of the needle electrodes relative to the orientation of the muscle fibers.

These data show that an electrotransfer device can employ either plate or needle
10 electrodes, regardless of the electrode orientation relative to the target tissue. Use of needle electrodes may be preferred to administer nucleic acids to muscles of large size to ensure that the total voltage is moderate, for example 100 V with placement of the needle electrodes within 0.5 cm for an electric field strength of 200 V/cm. However, the plate electrodes, which are non-invasive, may be preferred with small muscles, *e.g.*, the fingers, such as for delivery of a gene
15 therapy for arthritis.

EXAMPLE 17: ELECTROTRANSFER INTO DIFFERENT MUSCLES AND SPECIES

This Example illustrates that the electrotransfer device can be used to effect nucleic acid transfer into many different types of muscles in different species of animals.

20 The electrotransfer device was adjusted to provide the conditions for each species as defined in Table 8. The results are shown in Table 8 as well.

Table 8. Electrotransfer enhancement of nucleic acid transfection in various species and muscles

Species	Plasmid	electric field condition	Muscle Tibial cranial	Muscle Gastrocnémien	Muscle Rectus femoris	Muscle Triceps brachii	Muscle Quadriceps
Mouse	10 µg pXL3031	8 x 200 V/cm 20 msec, 2 Hz	x 28	x 196	x 342	x 1121	
Rat	150 µg pXL3031	8 x 200 V/cm 20 msec, 2 Hz	x 31			x 160	x 13,2
Rabbit	200 µg pXL2774	4 x 200 V/cm 20 msec, 1 Hz	x 25417			x 724	x 3595

5

The relative increase in the level of luciferase expression using an electrotransfer device relative to control (no electrotransfer) is indicated. The data are the average of 10 muscles per group. Luciferase activity was determined seven days after administration of the plasmid.

10 Electrotransfer was also tested in monkeys (*Macaca fascicularis*). Plasmid pXL3179 (Figure 11) comprising a gene encoding fibroblast growth factor 1 (acidic fibroblast growth factor) (FGF1 or aFGF) was derived from plasmid pXL2774 in which the human fibroblast interferon signal peptide was fused to cDNA for aFGF (sp-FGF1, Jouanneau *et al.*, 1991, *PNAS* 88:2893-2897) was introduced under control of the human CMV-IE promoter and the SV40 polyadenylation signal. aFGF expression was determined by immuno-histochemistry. The number of positive cells (cells expressing aFGF) were evaluated three days after intramuscular injection with 500 µg of plasmid pXL3179. The electric field conditions were 200 V/cm, 8 pulses of 20 msec each, at 1 Hz frequency. Controls were not treated with the electric field (electrotransfer -).

20 The results of this experiment are shown in Table 9. The data clearly demonstrate that aFGF protein expression could only be detected after use of an electrotransfer device to increase the efficiency of DNA transfer into the muscle tissues. Interestingly, no expression could be detected in the absence of electrotransfer under these conditions.

Table 9: Immunohistochemical analysis of aFGF expression in monkey muscle

	Electrotransfer -	Electrotransfer +
Triceps	0	0
Tibial cranial	0	30
Biceps	0	4
Quadriceps	0	30

Immunohistochemical analysis of expression of aFGF in different muscles of the monkey. The values indicate the number of positives three days after intramuscular injection of 500 µg of plasmid pXL3179 coding for aFGF, with (+) and without (-) application of an electric field.

10 EXAMPLE 18: ELECTROTRANSFER IN THE RAT DIAPHRAGM MUSCLE

The ability by use of an electrotransfer device of the invention to provide for long term, stable expression of a transgene has important implications in the treatment of degenerative diseases that affect function of the diaphragm, notably muscular dystrophy.

In these experiments, the diaphragm was rendered accessible by an incision along the sternum after anesthesia (mixture of 1 mg/kg largactyl and 150 mg/kg ketamine). The injection was made in the hemidiaphragm (50 µg of plasmid pXL2774 in 50 µl of NaCl 20 mM and glucose 5%). The plate electrodes were then placed one on either side of the plane of the diaphragm along the injection path at an inter-electrode distance of 1 mm. The electric field conditions used were as follows: 160 V/cm or 300 V/cm; 8 pulses of 20 mscd duration each; 1 Hz frequency. The electric field was applied to the muscle less than one minute after the injection. The incision in the animal was then closed.

The results are shown in Table 10.

Table 10: Electrotransfer into rat diaphragm muscle.

V/cm	0	160	300
RLU total	48	920	51
	± 33	± 474	± 29

The values for luciferase expression are the average ± S.E.M. of luciferase activity in millions of RLUs per muscle. N=12 for each group.

This example demonstrates a significant amelioration of expression of the transgene in the diaphragm after application 8 20 msec pulses at a field strength of 160 V/cm ($p < 0.003$ using

the Mann-Whitney non parametric test).

EXAMPLE 20: ELECTROTRANSFER OF A SECRETED ALKALINE PHOSPHATASE GENE

5 This example demonstrates the ability to transfect and express a gene encoding a secreted protein. Secreted proteins are used, for example, in a systemic gene therapy approach, and for generating an immune response (DNA vaccine). The secreted gene presented here was found in the circulation at an elevated concentration, and its presence was stable.

 In this example, the alkaline phosphatase-encoding plasmid pXL3010 (Figure 13) was
10 injected into one of the two tibial cranial muscles of an adult C57Bl/6 mouse. The plasmid pXL3010 was derived from ColE1 in which the gene coding for secreted alkaline phosphatase (SeAP) from pSEAP-basic (Clontech; Genbank accession no. CVU09660) was introduced under control of the CMV promoter (pCDNA3; Invitrogen, Netherlands) and the SV40 polyadenylation signal. The application of the electric field was performed under standard conditions, *i.e.*, 8
15 square pulses of 20 msec duration, 1 Hz frequency, and 200 V/cm applied 20 seconds after injection of the plasmid. Measurement of the concentration of the blood serum serum alkaline phosphatase was carried out on a blood sample from the eye (retro-orbital plexus puncture) 7 days later using a commercial chemiluminescence assay (Phosphalight, Tropix, Bedford, Massachusetts, US). The injection of a few muscles subjected or not to the electric field with a
20 non-encoding plasmid (ballast DNA) allowed verification of the absence of serum alkaline phosphatase that does not come from the expression of the transgene.

 The effect of improvement of the expression of the transgene by the application of an electric field under these conditions was clear for the various quantities of plasmid injected (Table 11). It was possible to reach high serum concentrations of alkaline phosphatase by increasing the
25 quantity of plasmid injected. This gain relative to a conventional transfection was maintained for a long period after injection in this experiment.

Table 11: Expression of SeAP from mouse muscle with and without electrotransfer

Plasmide pXL3010 μg	Plasmide pUC19 μg	Electrotransfert -	Electrotransfert +
0.1	0	0.03 ± 0.01 (n=5)	1.23 ± 0.21 (n=10)
0.3	0	0.05 ± 0.02 (n=5)	1.92 ± 0.33 (n=10)
1	0	0.16 ± 0.04 (n=5)	7.58 ± 1.18 (n=10)
10	0	1.52 ± 0.59 (n=10)	262.87 ± 54.97 (n=10)
400	0	15.64 ± 10.77 (n=5)	2203.11 ± 332.34(n=5)
0.1	9.9	0.088 ± 0.015 (n=5)	21.39 ± 3.54 (n=10)
0.3	9.7	0.90 ± 0.49 (n=5)	95.67 ± 16.15 (n=10)
1	9	0.26 ± 0.09 (n=5)	201.68 ± 32.38 (n=10)
10	0	0.21 ± 0.05 (n=10)	357.84 ± 77.02 (n=10)

5

The average value for SeAP in serum ± S.E.M. in ng/ml is reported in this table.

Injection of 400 μg of plasmid (injection of 100 μg DNA in 54 μl bilaterally and twice at a 30-minute interval) yielded a serum concentration of 2.2 μg/ml of alkaline phosphatase with electrotransfer compared to 0.016 μg for the controls. In addition, the use of ballast DNA, which makes it possible to work with a constant quantity of DNA regardless of the amount of plasmid (a total of 10 μg of DNA per mouse), further improves the levels of electrotransfection for a small quantity of plasmid pXL3010 (≤ 1 μg).

The kinetics of SeAP expression were followed as well. In this case, the dose of DNA was 15 μg was injected per muscle bilaterally (30 μg per mouse). The results are presented in Figure 9. One observes that, seven days after transfection, a significant and stable (at least for two months) serum concentration of SeAP as a result of the electrotransfer of pXL3010.

These results confirm that the transfer of nucleic acids in the muscle using an apparatus of the invention permits expression of high and stable levels of a secreted transgene. Thus, it is possible to use the muscle as an organ for production of a secreted protein of interest, as well as to direct expression of a therapeutic gene that acts directly on the muscle itself (such as the dystrophin gene or an angiogenic factor).

EXAMPLE 21: ELECTROTRANSFER OF A ERYTHROPOIETIN GENE

This example demonstrates that a therapeutic gene can be transferred to muscle using an apparatus of the invention, and that expression of the gene product effects a detectable and meaningful physiological response. In this case, the expression of erythropoietin can be detected, and the expressed protein induces an increase in the hematocrit of the recipient animal.

C57Bl/6 mice were injected in the tibial cranial muscle unilaterally with plasmid pXL3348 (Figure 16), comprising the gene coding for erythropoietin. The plasmid pXL3348 was derived from plasmid pXL2774 by introducing the murine erythropoietin gene (NCBI: 193086) under control of the human CMV-IE promoter and SV40 polyadenylation signal. The electric field (200 V/cm, 8 pulses of 20 msec duration, 1 Hz frequency) was applied immediately after injection of the plasmid.

The result of this experiment are reported in Table 12.

Table 12: Expression and its effect of erythropoietin

Plasmid	Serum level of erythropoietin (mIU/ml) at D7		Serum level of erythropoietin (mIU/ml) at D24	
	Electrotransfer -	Electrotransfer +	Electrotransfer -	Electrotransfer +
pXL3348 (1 µg)	0	3.0 ± 1.6	0	1.12 ± 0.8
pXL3348 (10 µg)	0.9 ± 0.9	61.8 ± 15.8	0	74.1 ± 28.9
pUC19 (1 µg)		0		0

Plasmid	Hematocrit % Increase at D7		Hematocrit % Increase at D24	
	Electrotransfer -	Electrotransfer +	Electrotransfer -	Electrotransfer +
pXL3348 (1 μ g)	38.5 \pm 0.5	35.0 \pm 3.6	50.8 \pm 2.3	81 \pm 0.5
pXL3348 (10 μ g)	32.0 \pm 3.2	26.0 \pm 4.1	69.0 \pm 5.1	83.0 \pm 1.0
PUC 19 (1 μ g)		30.8 \pm 2.3		43.2 \pm 0.9

Average value \pm S.E.M.; N=4 to 5 mice per group.

At D24, the injection of 1 μ g of plasmid was associated with a moderate increase of the hematocrit for mice conventionally transfected, and was very high for the electrotransfected mice. With 10 μ g of plasmid, the hematocrit increased for the control group. For the electrotransfected group, the hematocrit was clearly greater, with less variance. Similar results were observed with a lower amount of DNA (1 μ g).

10 EXAMPLE 22: ELECTROTRANSFER OF VEGF (VASCULAR ENDOTHELIAL GROWTH FACTOR) GENE

C57Bl/6 or SCID mice were injection in the tibial cranial muscle bilaterally with 15 μ g of plasmid pXL3212 (Figure 11), a VEGF-encoding plasmid pCOR hVEGF. Plasmid pXL3212 was derived from plasmid pXL2774 by introducing the cDNA coding for VEGF (Genbank accession no. HUMEGFAA) under control of the human CMV-IE promoter and the SV40 polyadenylation signal. The electrotransfection was carried out using a commercial electropulser (Jouan) at a rate of 8 pulses of 20 msec duration, 200 V/cm, at a frequency of 2 Hz. Blood samples were taken from the retro-orbital plexus in dry tubes. Blood samples were taken one day before and 7 days after injection of the plasmid. The immunoenzymatic quantitation of human VEGF was made using the Quantikine kit (R&D System). A supplemental human VEGF series was performed in mouse serum. The results for that series are shown in Table 13.

Table 13: Expression of human VEGF in mouse serum

Mouse strain	Day	Transfection method	Human VEGF (ng/L)
C57BL6	D-1	simple injection	not detectable
C57BL6	D+7	electrotransfer	393 + 110
SCID	D-1	simple injection	not detectable
SCID	D+7	electrotransfer	99 + 26

5 Serum concentration (ng/liter) of VEGF in C57Bl/6 and SCID mice. Control mouse serum was obtained from mice one day prior to injection of the plasmid.

EXAMPLE 23: ELECTROTRANSFECTION OF A CLOTTING FACTOR IX GENE

The experimental conditions were the same as in Example 22, except that 15 µg of the clotting factor IX-encoding plasmid pCor hFIX (pXL3388; Figure 12) was injected per muscle in C57BL6 or SCID mice. The pXL3388 plasmid was derived from plasmid pXL2774 by introducing the cDNA encoding human factor IX (Christmas factor; Genbank accession no. HUMFIXA) under control of the CMV-IE promoter and SV40 polyadenylation signal. The electrotransfer conditions were as follows: 8 pulses of 20 msec duration at 200 V/cm, 2 Hz frequency. Factor IX levels were measured seven days after injection of the plasmid. Blood samples were taken from the retro-orbital plexus in tubes containing trisodium citrate, and the tubes stored in ice.

The following table (Table 14) shows that the human factor IX was found only in the blood of mice C57BL6 and SCID whose tibial cranial muscles were injected with pXL3388 plasmid and subjected to the application of an electric field using an electrotransfer apparatus of the invention.

Table 14: Expression of human factor IX

Mouse strain	injection	Electrotransfer	Human factor IX (µg/L)
C57BL/6	pXL3388	+	69 + 12
C57BL/6	pXL3388	-	not detectable
C57BL/6	NaCl 0.9 %	+	not detectable
SCID	pXL3388	+	66 + 5
SCID	pXL3388	-	not detectable

25 The concentration of factor IX in C57Bl/6 and SCID mice.

Human factor IX is not detectable in mouse blood in the absence of use of an

electrotransfer apparatus of the invention.

EXAMPLE 24: ELECTROTRANSFER OF AN ACIDIC FIBROBLAST GROWTH FACTOR (aFGF) GENE

5 The experimental conditions are like those in Example 22, except for the fact that 15 µg of the FGF-encoding plasmid pCor CMV a FGF (pXL3096; Figure 14) was injected per muscle in C57BL6 or SCID mice. The pXL3096 plasmid was derived from plasmid pXL2774 by introduction of a triple helix forming sequence (TH; Wils *et al.*, 1997, *Gene Ther.* 4:323-330) in which the gene coding for a fusion between the signal peptide of human fibroblast interferon and the cDNA coding for FGF1 (sp-FGF1; Jouanneau *et al.*, *supra*) under control of the CMV-IE promoter, followed by the transcribed, non-translated leader sequence of HSV thymidine kinase, and the SV40 polyadenylation signal sequence. The following electrotransfer conditions were employed: 8 pulses of 20 msec duration, 200 V/cm, 2 Hz frequency. The presence of the FGF was shown by immunohistochemistry.

15 Results for transfection of C57BL/6 mice are shown in Figure 10, and result for the SCID mice are shown in Table 15. The number of fibers expressing FGF in randomly-selected sections was always clearly superior for the electrotransfected muscles than for the control muscles, which received only an injection of the pXL3096 plasmid alone. The expression of FGF after electrotransfection reaches a maximum at D8. On D21 and D35, the presence of FGF for the control groups is virtually undetectable whereas a large number of positive fibers were observed in the electrotransfected groups.

Table 15: Expression of aFGF in SCID mice

	Electrotransfer	Tibial cranial (left)	Tibial cranial (right)
pXL3096 (15µg)	+	600	450
	+	700	300
pXL3096 (15µg)	-	3	0
	-	3	0
	-	0	0
pXL3096 (1.5µg)	+	80	70
	+	20	35
	+	110	100
pXL3096 (1.5µg)	-	0	0
	-	0	1

The number of aFGF positive fibers, detected by immunohistochemistry, in a muscle section were determined for individual mice. The muscle sections were obtained from the middle of the muscle.

- 5 The expression of aFGF, as determined by the number of positive fibers revealed by immunohistochemistry, was detected almost exclusively in mice that had received treatment with an electrotransfer apparatus. Moreover, aFGF expression was detectable at the lower DNA dose as well as the higher dose.

10 EXAMPLE 25: ELECTROTRANSFER OF A NEUROTROPHIC FACTOR-3 (NT3) GENE

- Five-week-old C57Bl/6 and baby *Xt/pm*n mice were injected unilaterally in the tibial cranial muscle with 12.5 µg of the plasmid pXL3149 (Figure 14) which encodes for the neurotrophic factor NT3. The *pm*n mice are a model for amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease) characterized by an early and rapid degeneration of motoneurons and by an average life expectancy of about 40 days. Plasmid pXL3149 was derived from plasmid pXL2774 by introduction of the murine NT3 gene (Genbank accession no. MMNT3) under control of the human CMV-IE promoter and the SV40 polyadenylation signal. The expression of the NT3 was studied in the supernatant prepared by centrifugation (12,000 x g) of the ground muscle in PBS buffer 7 days after the treatment of the mice, and quantified by an ELISA assay (Promega Kit).

- 20 With the C57Bl/6 mice received injections of 12.5 µg of plasmid DNA. Half of the mice were subjected to an electric field (250 V/cm with 4 pulses of 20 ms at a frequency of 1 Hz) immediately after injection. The respective 95% confidence intervals calculated for an average of 20 muscles are 77±11 pg/muscle when there was no electrotransfer and 2.7±0.9 ng/muscle with electrotransfer. The endogenous NT3 level was not determined.

- 25 Similar data were found for expression of NT3 in 4 to 5 day old *Xt/pm*n heterozygous mice. These mice received injections of 130 µg of DNA per animal after multisite injection in different muscles (gastrocnemien, 25 µg; tibial cranial muscle, 12.5 µg). The following electrotransfer conditions were employed: 4 pulses of 20 msec duration, 500 V/cm, 1 Hz. NT3 was detected in these mice seven days after administration of the plasmid and application of the electric field. The results of this experiment are reported in Table 16.
- 30

Table 16: Expression of NT3 in *Xt/pm* mice

Electrotransfer	NaCl 0,9 % (control)		pXL3149	
	-	+	-	+
Plasma	0 (n=2)	0 (n=2)	46 ± 10 (n=4)	1599 ± 639 (n=4)
Muscle gastrocnemien	3619 ± 102 (n=4)	1619 ± 150 (n=2)	3647 ± 1078 (n=8)	19 754 ± 3818 (n=8)
Muscle tibial cranial	1415 ± 363 (n=4)	1453 ± 375 (n=2)	1400 ± 155 (n=8)	16 826 ± 3135 (n=8)

Average values of NT3 ± S.E.M. (pg per muscle or pg/ml of plasma).

Under the experimental conditions tested here, a basal level of NT3 was detected in the gastrocnemien and tibial cranial muscles. Under standard DNA transfer conditions, injection of plasmid pXL3149 increases the level of NT3 expression. When an apparatus of the invention is used, a very large increase in the amount of NT3 in the tissue and plasma is observed. Thus, for any quantity of DNA plasmid that is administered, utilization of an apparatus of the invention to increase transfection efficiency greatly increases the amount of transgene product expressed, both in the muscle and in plasma. This increase is especially important for NT3 expression, to achieve a neurotrophic gene therapy.

EXAMPLE 26: ELECTROTRANSFER OF A FOR HUMAN GROWTH HORMONE GENE

C57Bl/6 mice received an injection of plasmid pXL3353 (Figure 15) or plasmid pXL3354 (Figure 15) (10 µg) unilaterally in the tibial cranial muscle. Plasmid pXL3353 is derived from plasmid pXL2774 by introduction of a genomic human growth hormone gene (fragment XbaI/Sph of hGH that extends from the transcription initiation signal to a BamHI site, which is 224 basepairs after the polyadenylation signal) under control of the human CMV-IE promoter and the SV40 polyadenylation signal. The hGH cDNA was obtained by reverse transcription from a human pituitary mRNA library after 30 cycles of amplification using the following primers:

5' complementary oligo:

5'- GGGTCTAGAGCCACCATGGCTACAGGCTCCCGGAC -3'

This oligonucleotide contains a kozak XbaI sequence.

3' complementary oligonucleotide:

5'- GGGATGCATTTACTAGAAGCCACAGCTGCCTC-3'

This oligonucleotide contains an NsiI site and the stop codon.

The amplified fragment was cloned into plasmid pCR2.1 (TA Cloning Kit, Invitrogen) and sequenced. An XbaI/NsiI fragment of 681 basepairs containing the hGH cDNA was ligated with the XbaI/NsiI fragment of pSL3353 to generate plasmid pXL3354.

5 Electrotransfer conditions were as follows: 200 V/cm; 8 pulses of 20 msec duration; 1 Hz frequency. The electric field was applied immediately after injection of the plasmid DNA. The presence of hGH was detected seven days after treatment of the mice in ground muscle supernatant in buffered PBS after centrifugation at 12,000 x g. The quantity of hGH was measured by ELISA (Boehringer Mannheim).

10 The results of this experiment are reported in Table 17.

Table 17: Expression of human growth hormone

Electrotransfer	Injection genomic hGH (pXL3353)		Injection hGH cDNA (pXL3354)	
	-	+	-	+
Muscle	87,1 ± 9,3	1477,6 ± 67,6	2820,0 ± 487,5	15739,1 ± 915,5
Tibial Cranial	(n=10)	(n=10)	(n=10)	(n=10)

Average value of hGH expression (picogram/muscle) ± S.E.M.

15

These results show that use of an electrotransfer apparatus of the invention permits much higher expression of human growth hormone. This increase in expression is observed whether a genomic clone or cDNA encoding hGH was administered.

20 EXAMPLE 27: ELECTROTRANSFER OF VACCINE TRANSGENES

This Example reports that an apparatus for electrotransfer of the invention enhances delivery of genes for gene (or DNA) vaccination. In this example, the following products were used: VR-HA, a plasmid DNA including the gene of the hemagglutinin of the flu virus (strain A/PR/8/34). mVRgBDT is a plasmid DNA including the gene of the glycoprotein B (gB) of the
25 human cytomegalovirus (Towne strain). The other products are available from commercial suppliers: Ketamine, Xylazine and physiological sodium chloride solution (NaCl 0.9%). The experiment was performed in 9-week-old female mice Balb/c. Mice originating in different cages were distributed randomly before the experiment (randomization).

An oscilloscope and a commercial generator of electrical pulses (rectangular or square)
30 (Electropulser PS 15, Jouan, France) were used. The electrodes used were stainless steel flat electrodes spaced 5 mm apart.

The mice were anesthetized using a mixture of ketamine and xylazine. The plasmid solution (50 μ l of a solution at 20 μ g/ml or 200 μ g/ml in NaCl 0.9%) was injected longitudinally through the skin into the tibial cranial muscle of the left leg using a Hamilton syringe. The two electrodes are coated with a conductive gel and the injected leg was placed between the electrodes in contact with them. The electrical pulses were applied perpendicularly to the axis of the muscle using a square pulse generator, 20 seconds after the injection. An oscilloscope enabled monitoring the electric field: 200 V/cm, duration of 20 ms, frequency of 1 Hz for 8 consecutive pulses.

For the evaluation of the stimulation of the immune response, the following immunization protocol was followed:

- 10 D 0 → pre-immune serum sample was taken
- D 1 → first injection, with or without electrotransfer
- D 21 → immune serum sample was taken
- D 22 → booster injection, with or without electrotransfer
- D 42 → immune serum sample was taken
- 15 D 63 → immune serum sample was taken

The blood serum samples were taken from the retro-orbital sinus. The amounts of specific antibodies were determined by ELISA. Each experimental condition was tested at 10 points using 10 animals injected unilaterally.

The results of antibody titers directed against flu hemagglutinin are presented in Table 18.

20

Table 18: Anti-flu hemagglutinin antibody response

	Electrotransfer	D0	D21	D42	D63
VR-HA (1 μ g)	-	< 50	132 \pm 739	1201 \pm 4380	1314 \pm 2481
VR-HA (1 μ g)	+	< 50	1121 \pm 1237	10441 \pm 7819	8121 \pm 5619
(p)			(0.0135)	(0.0022)	(0.0033)
VR-HA (10 μ g)	-	< 50	781 \pm 666	5113 \pm 16015	4673 \pm 8238
VR-HA (10 μ g)	+	< 50	4153 \pm 2344	74761 \pm 89228	41765 \pm 52361
(p)			(0.0002)	(0.0005)	(0.0007)

25 Antibody titers directed against flu hemagglutinin, obtained after injection of 1 or 10 μ g of VR-HA DNA in the absence (-) or presence (+) of an electric field provided by an electrotransfer apparatus. The results are the geometric average of ten animals per group (N=8 for the group injected with 1 μ g of DNA, and exposed to the electric field, and tested at D63) \pm standard error. The value (p) was obtained by comparison two by two of the groups injected with

DNA, then treated or not with the electric field using the non parametric Mann-Whitney test.

These results show that the antibody titer directed against flu hemagglutinin in greatly increased, by about a factor of ten, in the group treated with the electrotransfer apparatus. Indeed, mice that received only 1 μ g of the DNA, and treated with the electrotransfer apparatus, had a greater anti-hemagglutinin titer than mice that received 10 μ g of DNA, but were not treated with the electric field.

The results of the antibody response directed against CMV glycoprotein B is presented in Table 19.

Table 19: Anti-CMV glycoprotein B antibody response

	Electrotransfer	D0	D21	D42	D63
VR-gB (10 μ g)	-	< 50	73 \pm 138	755 \pm 1766	809 \pm 1363
VR-gB (10 μ g)	+	< 50	200 \pm 119	3057 \pm 1747	2112 \pm 1330
(p)			(0.0558)	(0.0108)	(0.0479)

Antibody titers directed against CMV glycoprotein B, obtained after injection of 10 μ g of VR-gB DNA in the absence (-) or presence (+) of an electric field provided by an electrotransfer apparatus. The results are the geometric average of ten animals per group (N=9 for the exposed to the electric field) \pm standard error. The value (p) was obtained by comparison two by two of the groups injected with DNA, then treated or not with the electric field using the non parametric Mann-Whitney test.

These results show that the anti-gB titer is increased by a factor of four by day 42 (D42) in the group treated with an electric field compared to the control group. In addition, as observed previously, the variation (standard error) is greatly reduced for mice treated with the electrotransfer apparatus compared to untreated (control) mice.

EXAMPLE 28: ELECTROTRANSFER APPARATUS FOR TRANSFECTION OF TUMOR CELLS

The following Example illustrates use of an electrotransfer apparatus to enhance delivery of nucleic acids into tumor tissue. In particular, by modifying an electrotransfer apparatus of the invention to provide higher voltages than are preferred for electrotransfer of nucleic acids into muscle, efficient transfection of tumor cells (and most other cells) *in vivo* can be effected. This example demonstrates effects of electrotransfer on different tumors of either human origin implanted on nude (immunodeficient) mice, or of murine origin implanted on C57B1/6

(immunocompetent) mice. The effects of low-intensity electric-field pulses have been demonstrated: A) on plasmid DNA transfection by intratumoral injection, and B) on secretion of a protein encoded by a transgene into plasma following intratumoral injection.

5

Materials and Methods

Tumor grafts were implanted on one side of either female nude or C57Bl/6 mice weighing 18-20 g. Human lung carcinoma (H1299) or colon adenocarcinoma (HT29) tumors of 20 mm³ were implanted in nude mice. Murine fibrosarcoma (LBP) cells (10⁶ cells), or melanoma (B16) or lung carcinoma (3LL) tumors (20 mm³) were implanted in C57Bl/6 mice. The mice were
10 classified according to the size of their tumors and divided into homogeneous lots.

The mice were anesthetized with a mixture of ketamine and xylazine. Either plasmid pXL3031 (cytoplasmic luciferase) or pXL3010 (secreted alkaline phosphatase) were injected intratumorally after the tumors reached the target volume. The plasmid solution (40 µl of a 250 µg/ml solution of DNA in 20 mM NaCl, 5% glucose) was injected lengthwise into the center of
15 the tumor with a Hamilton syringe. The lateral surfaces of the tumor were coated with a conductive gel and the tumor was placed between the two electrodes. Electrical pulses were applied using a square pulse generator, 20 to 30 seconds after the injection. An oscilloscope controlled the voltage intensity, the duration in milliseconds and the frequency in hertz of the 8 pulses delivered at 200 to 800 volts/cm, 20 msec and 1 hertz. An oscilloscope and a commercial
20 electric-pulse (rectangular or square) generator (Electro-pulsateur PS 15, Jouan, France) were used. The electrodes were stainless steel plate electrodes separated by 0.45 to 0.7 cm.

To evaluate tumoral transfection with luciferase, the mice (generally 10 mice per experimental group, depending on conditions) were euthanized 2 days after the injection of the plasmid. The tumors were removed, weighed, and crushed in a lysis buffer. The suspension
25 obtained was centrifuged to obtain a clear supernatant. Luciferase activity was measured in 10 µl of supernatant using a commercial luminometer in which the substrate was added automatically. The results were expressed in total RLUs (Relative Light Units) per tumor.

Plasma levels of secreted alkaline phosphatase (SeAP) were measured as described in Example 20, *supra*, at days 1, 2, and 8 (D1, D2, D8) after injection of the DNA.

Results and Discussion

Electrotransfer into a human lung carcinoma tumor. In a first experiment, the conditions generally used for intramuscular gene electrotransfer were used: an electric field of 200 v/cm, 8 pulses at a frequency of 1 hertz, and the results obtained were compared with those obtained at higher voltages ranging from 300 to 500 volts/cm. The purpose of a second experiment was to determine the optimal voltage conditions that must be applied to obtain maximum transfection, or voltages ranging from 400 to 800 volts/cm. The results are shown in Table 20.

Table 20. Electrotransfer into a human lung carcinoma tumor

RLU/tumor				
Volt/cm	Trial 1		Trial 2	
	Average	SEM	Average	SEM
0	32 807 758	6 790 565	44 723 317	10 163 318
200	129 744 454	39 119 425		
300	585 033 326	134 810 534		
400	5 266 632 345	1 473 785 460	8 488 242 519	3 881 651 201
500			14 201 644 540	6 162 551 269
600			7 401 041 930	5 323 128 047
800			11 884 115 963	4 048 340 474

Plasmid pXL3031 was injected into H1299 human lung carcinoma tumors that had reached the target volume of 200-300 mm³ in female nude mice. Average values of luciferase expression with the SEM are reported.

According to Table 20, it can be seen that relative to the control group, where DNA is injected without subsequent application of an electric field:

- gene transfer is increased in a manner that is dependent on the applied voltage of 200 to 400 volts/cm until it reaches a plateau corresponding to the maximum transfection obtained, starting at 500 volts/cm;
- at higher voltages (600 and 800 volts/cm), cutaneous or deeper burns were obtained, respectively; however, transgene expression was not decreased.

- amplification of gene transfer by electrotransfer was on the order of 240 to 320-fold.

Electrotransfer into a human colon adenocarcinoma tumor. The results of the two experiments are illustrated in Table 2. Compared to the control groups without electrotransfer, the application of an electrical field with an intensity of 600 volts/cm made it possible to reach an optimal rate of transfection regardless of the transfection level without electrotransfer. Transfection was improved by a factor of 6 to 23-fold, respectively, and it was relatively similar from 400 to 600 volts/cm.

10 Table 21: Electrotransfer into a human colon adenocarcinoma tumor

RLU/tumor				
	Trial 1		Trial 2	
Volt/cm	Average	SEM	Average	SEM
0	4 043 062	1 827 237	634 999	338 311
400	16 037 136	5 420 572		
500	14 096 640	7 629 212	5 537 359	3 571 433
600	24 223 872	9 217 062	14 607 850	6 392 841

Plasmid pXL3031 was injected into HT29 human colon adenocarcinoma tumors that had reached the target volume of 100-200 mm³ in female nude mice. Average values of luciferase expression with the SEM are reported. The inter-electrode distance in this experiment was 0.45 cm.

Electrotransfer into a murine fibrosarcoma tumor. The results of two experiments are illustrated in Table 22. Compared to the control groups without electrotransfer, the application of an electrical field with an intensity of 300 to 600 volts/cm improved gene transfer by a factor of 30 to 70-fold, regardless of the voltage applied.

Table 22: Electrotransfer into a murine fibrosarcoma tumor.

RLU/tumor				
	Trial 1		Trial 2	
Volt/cm	Average	SEM	Average	SEM
0	581 270	348 645	394 922	129 395
300	26 296 355	14 811 826	11 579 942	4 615 332
400	42 498 832	31 152 744	10 431 574	3 495 118
500	16 966 612	12 754 188	6 034 954	1 818 465
600			10 952 214	7 093 932

- 5 Plasmid pXL3031 was injected into murine LPB fibrosarcoma tumors that had reached the target volume of 100-200 mm³ in female C57Bl/6 mice. Average values of luciferase expression with the SEM are reported.

Electrotransfer of murine melanoma tumors. The results are illustrated in

- 10 Table 23. Compared to the control group without electrotransfer, the application of an electrical field with an intensity of 500 volts/cm improves gene transfer by a factor of 24-fold.

Table 23: Electrotransfer of murine melanoma tumors.

RLU/tumor		
Volt/cm	Average	SEM
0	1 318 740	667 588
300	14 275 486	7 625 262
500	32 249 218	12 605 041
600	17 215 505	6 241 666

- 15 Plasmid pXL3031 was injected into murine B16 melanoma tumors that had reached the target volume of 200-300 mm³ in female C57Bl/6 mice. Average values of luciferase expression with the SEM are reported.

Electrotransfer of a murine lung carcinoma tumor. The results of this experiment are

- 20 reported in Table 24.

Table 24: Electrotransfer of a murine lung carcinoma tumor

Volt/cm	RLU/tumor	
	Average	SEM
0	121 080	37 322
300	3 715 877	2 936 873
500	470 499 612	237 588 443
600	53 275 350	23 857 181

- 5 Plasmid pXL3031 was injected into murine 3LL lung carcinoma tumors that had reached the target volume of 30 mm³ after 5 days of growth in female C57Bl/6 mice. Average values of luciferase expression with the SEM are reported.

- 10 The application of an electrical field with an intensity of 500 volts/cm optimally improves gene transfer by a factor of 3885-fold. These impressive results are related to the fact that these tumors are very poorly transfectable by naked DNA under conditions without electrotransfer compared to the other tumors that were tested previously.

- 15 *Electrotransfer of a secreted transgene into a human lung carcinoma tumor.* The results of this experiment are shown in Table 25.

Table 25: Electrotransfer of a secreted transgene into a human lung carcinoma tumor

Evaluation time	Plasma alkaline phosphatase levels	
	0 Volt/cm (AVG \pm SEM)	500 Volts/cm (AVG \pm SEM)
D1	1.42 \pm 0.07	8.90 \pm 1.74
D2	1.40 \pm 0.01	9.04 \pm 1.55
D8	1.31 \pm 0.01	1.67 \pm 0.12

- 20 Plasmid pXL3010 (expressing SeAP) was injected into human H1299 lung carcinoma tumors that had reached the target volume of 200-300 mm³ in female nude mice. Average values of luciferase expression with the SEM are reported. A single electric field of 500 V/cm was applied, and the level of SeAP in plasma detected 1, 2, and 8 days after injection of the plasmid.

- 25 The results of this experiment demonstrate a dramatic, transient increase in the level of SeAP in plasma after transfection of tumor cells under electrotransfer conditions. Administration

of a immunostimulatory gene, such as GM-CSF or IL-2, to tumors is likely to provide for an effective amount of cytokine production. Moreover, these data, in combination with the luciferase expression data reported above, suggest that administration of a secreted cytokine with a suicide gene, such as HSV-thymidine kinase, would result in a robust anti-tumor response. Alternatively, 5 the data with SeAP suggest that electrotransfer-mediated transfection with an anti-angiogenesis gene, such as the amino terminal fragment of urokinase (ATF) or angiostatin (or endostatin) would also be an effective tumor gene therapy.

The data further demonstrate that an apparatus for electrotransfer of therapeutic genes into tumor cells provides an optimum electric field strength of between 400 and 600 Volts/cm, 10 with a likely optimum of $500 \text{ V/cm} \pm 10\%$ (*i.e.*, 450-550 V/cm).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying 15 figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by 20 reference in their entireties.

WHAT IS CLAIMED IS:

1. A system for *in vivo* nucleic acid transfer into cells of multicellular eukaryotic organisms, in which the tissue cells are brought in contact with the nucleic acid to be transferred
5 by direct administration into the tissue or by topical or systemic administration, and in which transfer is ensured by application to the tissue of one or more electric pulses of intensity ranging between 1 and 600 volts/cm, comprising:
 - a) an electrical pulse generator, wherein the electrical pulse generator produces electric pulses with pulse times of greater than 1 millisecond and of intensity ranging
10 between 1 and 600 volts/cm at a frequency of between 0.1 and 1,000 Hz; and
 - b) electrodes connected to the electrical pulse generator for generating an electric field in a tissue in contact with the electrodes.
2. The system according to claim 1, wherein the electrical pulse generator produces pulses of intensity ranging between 1 and 400 volts/cm.
- 15 3. The system according to claim 1, wherein the electrical pulse generator produces pulse of intensity ranging between 30 and 300 volts/cm.
4. The system according to claim 1, wherein the electrical pulse generator produces pulse times of greater than 10 milliseconds.
5. The system according to claim 1, wherein the electrical pulse generator produces
20 between 2 and 1,000 pulses.
6. The system according to claim 1, wherein the electrical pulse generator produces pulses irregularly in relation to one another, whereby a function describing the intensity of the field dependent on time of an pulse is variable.
7. The system according to claim 6, wherein the integral of the function describing
25 variation of the electric field with time exceeds 1 kV•msec/cm.
8. The system according to claim 7, wherein the integral exceeds or is equal to 5 kV•msec/cm.
9. The system according to claim 1, wherein the electrical pulse generator produces pulses selected from the group consisting of square-wave pulses, exponential decay waves, oscillating
30 unipolar waves of limited duration, and oscillating bipolar waves of limited duration.
10. The system according to claim 1, wherein the electrical pulse generator produces square-wave pulses.
11. The system according to claim 1, wherein an electrode is an external electrode for placement on a tissue to be treated.

12. The system according to claim 1, wherein an electrode is an internal electrode implantable in a tissue to be treated.
13. The system according to claim 1, wherein an electrode is an external electrode for placement on a tissue to be treated, and an electrode is an internal electrode implantable in the
5 tissue to be treated.
14. The system according to claim 13, wherein the external electrode is dimensioned to contact an external portion of a subjects body in close proximity to a large muscle.
15. The system according to claim 14, wherein the electrode is a flat plate electrode.
16. The system according to claim 14, wherein the electrode is a semi-cylindrical plate
10 electrode.
17. The system according to claim 1, wherein an electrode is an intra-arterial or intravenous electrode.
18. The system according to claim 12, wherein the internal electrode is an injector system making possible the simultaneous administration of nucleic acids and of the electric field.
- 15 19. The system according to claim 18, wherein an electrode is an external electrode for placement on the tissue to be treated.
20. The system according to claim 1, wherein the electrode is a stainless steel electrode.
21. An improved apparatus for *in vivo* nucleic acid transfer into cells of multicellular eukaryotic organisms, wherein the apparatus comprises means for generating an electric pulse
20 connected to electrodes for generating an electric field in a tissue *in vivo*, wherein the improvement comprises adapting the means for generating an electric pulse to produce pulse times of greater than 1 millisecond and of intensity ranging between 1 and 600 volts/cm at a frequency of between 0.1 and 1,000 Hz.
22. The apparatus of claim 21, wherein the means for generating an electric pulse generates
25 pulses of intensity ranging between 1 and 400 volts/cm.
23. The apparatus of claim 21, which is a flexible catheter apparatus.
24. The apparatus of claim 21, which is an apparatus for implanting nucleic acids into tissues by a tissue penetrating electrode.
25. The apparatus of claim 24, wherein the tissue penetrating electrode is a needle.
- 30 26. The apparatus of claim 21, which is an apparatus for transferring nucleic acids into cells of a surface tissue of a subject.
27. The apparatus of claim 21, wherein the means for generating an electric pulse is adapted to produce pulses ranging between 1 and 600 volts/cm by modifying the voltage gate not to exceed a voltage corresponding to 600 volts/cm.

28. The apparatus of claim 27, wherein the voltage is set at a constant voltage and the electrodes are set at a constant spacing distance.
29. The apparatus of claim 21, wherein the means for generating an electric pulse is adapted to produce pulses ranging between 1 and 600 volts/cm by labeling the device not to exceed a
5 voltage corresponding to 600 volts/cm.
30. The apparatus according to claim 21, wherein the means for producing an electrical pulse produces pulse of intensity ranging between 30 and 300 volts/cm.
31. The apparatus according to claim 21, wherein the means for producing an electrical pulse produces a pulse of intensity ranging from between 400 and 600 volts/cm.
- 10 32. The apparatus according to claim 21, wherein the means for producing an electrical pulse produces pulse times of greater than 10 milliseconds.
33. The apparatus according to claim 21, wherein the means for producing an electrical pulse produces between 2 and 1,000 pulses.
34. The apparatus according to claim 21, wherein the means for producing an electrical
15 pulse produces pulses irregularly in relation to one another, whereby a function describing the intensity of the field dependent on time of an pulse is variable.
35. The apparatus according to claim 21, wherein the means for producing an electrical pulse produces pulses selected from the group consisting of square-wave pulses, exponential decay waves, oscillating unipolar waves of limited duration, and oscillating bipolar waves of
20 limited duration.
36. The apparatus according to claim 21, wherein the means for producing an electrical pulse produces square-wave pulses.
37. The apparatus according to claim 21, wherein the electrode is a stainless steel electrode.

Figure 1: Effects of electric impulses of high field intensity on the transfection of plasmid DNA pxi 2774 in the cranial tibial muscle in the mouse.
Mean values \pm SEM.

A. Logarithmic scale

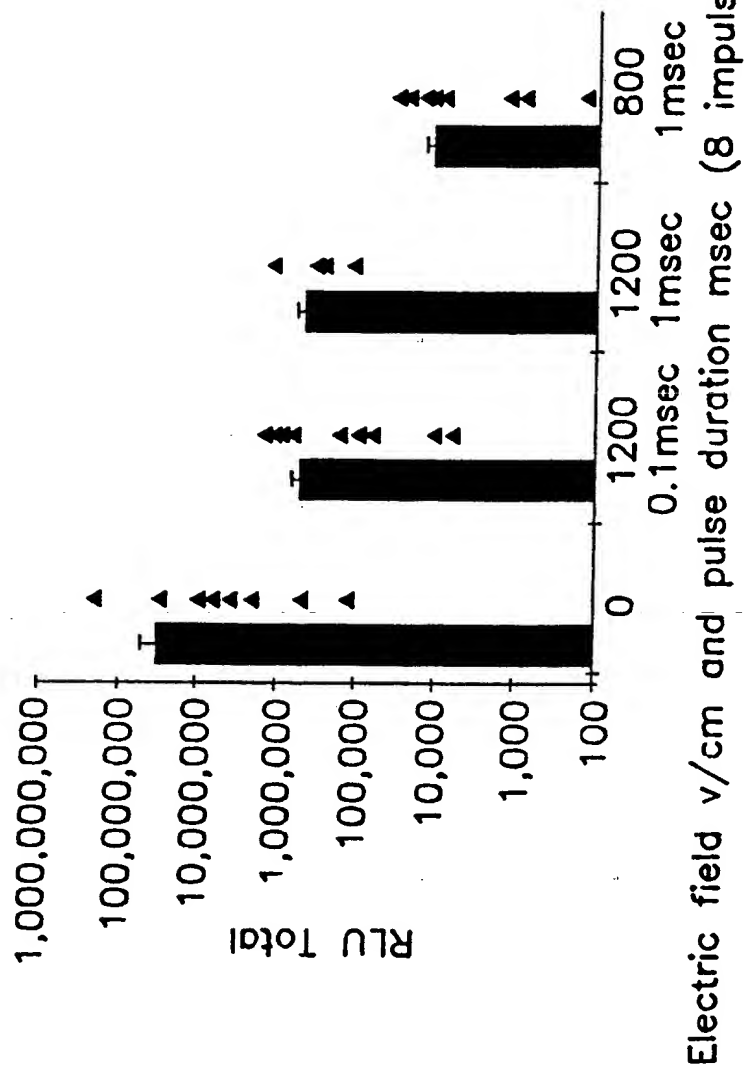


Figure 1a

**Figure 1: Effects of electric impulses of high field intensity on the transfection of plasmid DNA p_{GL2774} in the cranial tibial muscle in the mouse.
Mean values \pm SEM.**

B. Linear scale

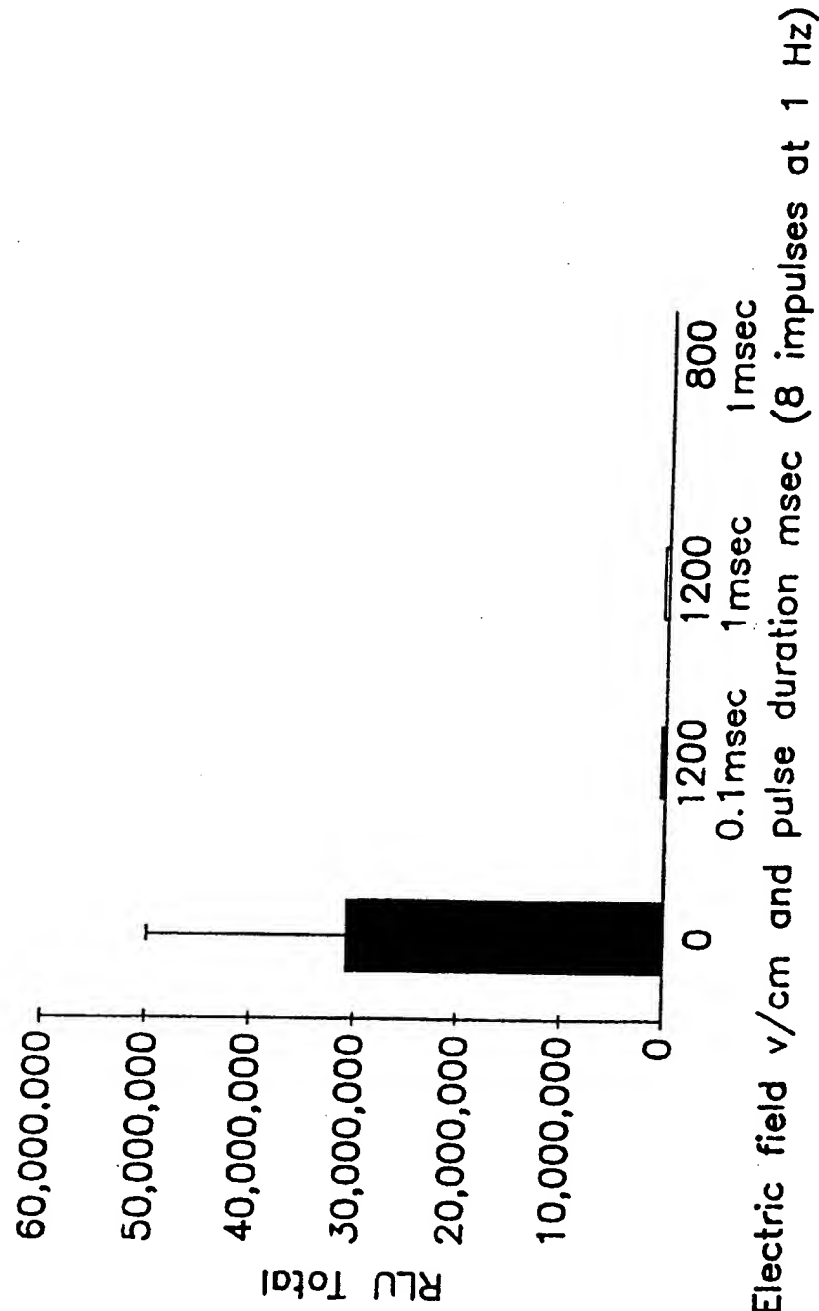


Figure 1b

Figure 2: Effects of electric impulses of intermediate field intensity on the transfection of plasmid DNA p_{GL2} 2774 in the cranial tibial muscle in the mouse.
Mean values \pm SEM.

A. Logarithmic scale

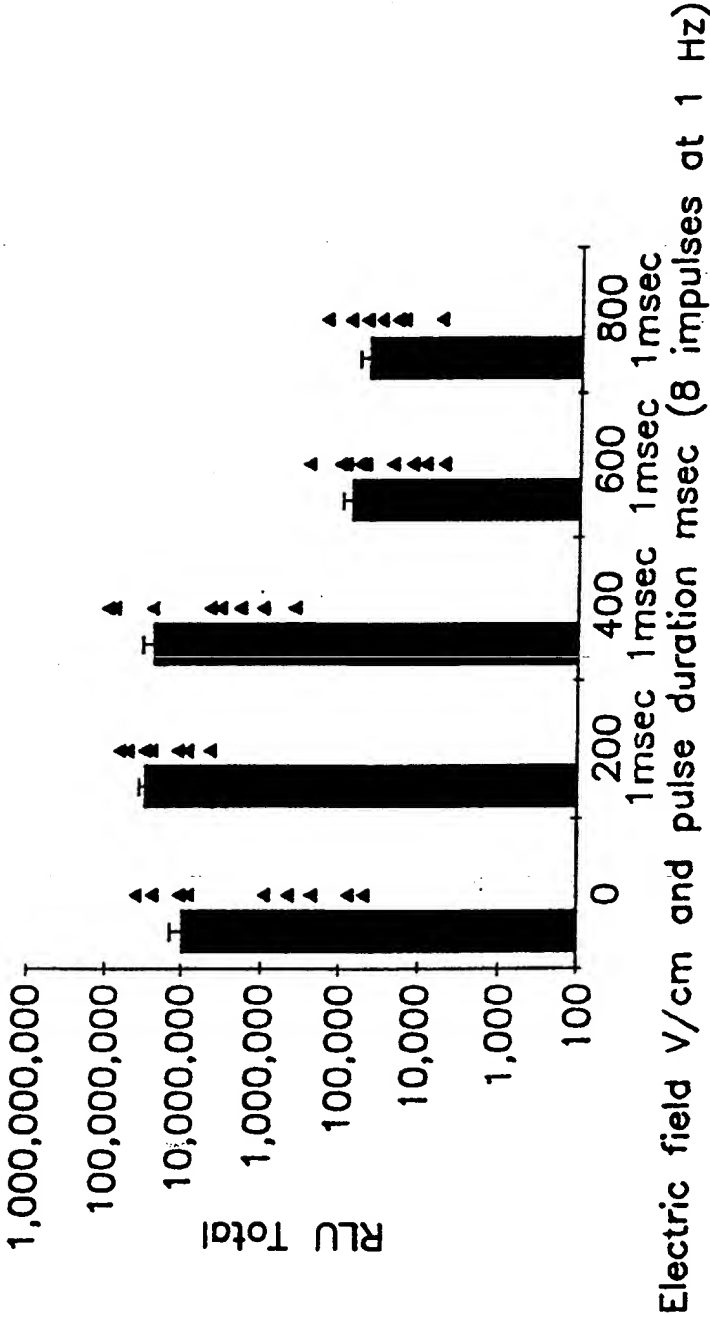


Figure 2a

Figure 2: Effects of electric impulses of intermediate field intensity of plasmid DNA p_{xl} 2774 in the cranial tibial muscle in the mouse. Mean values \pm SEM.

B. Linear scale

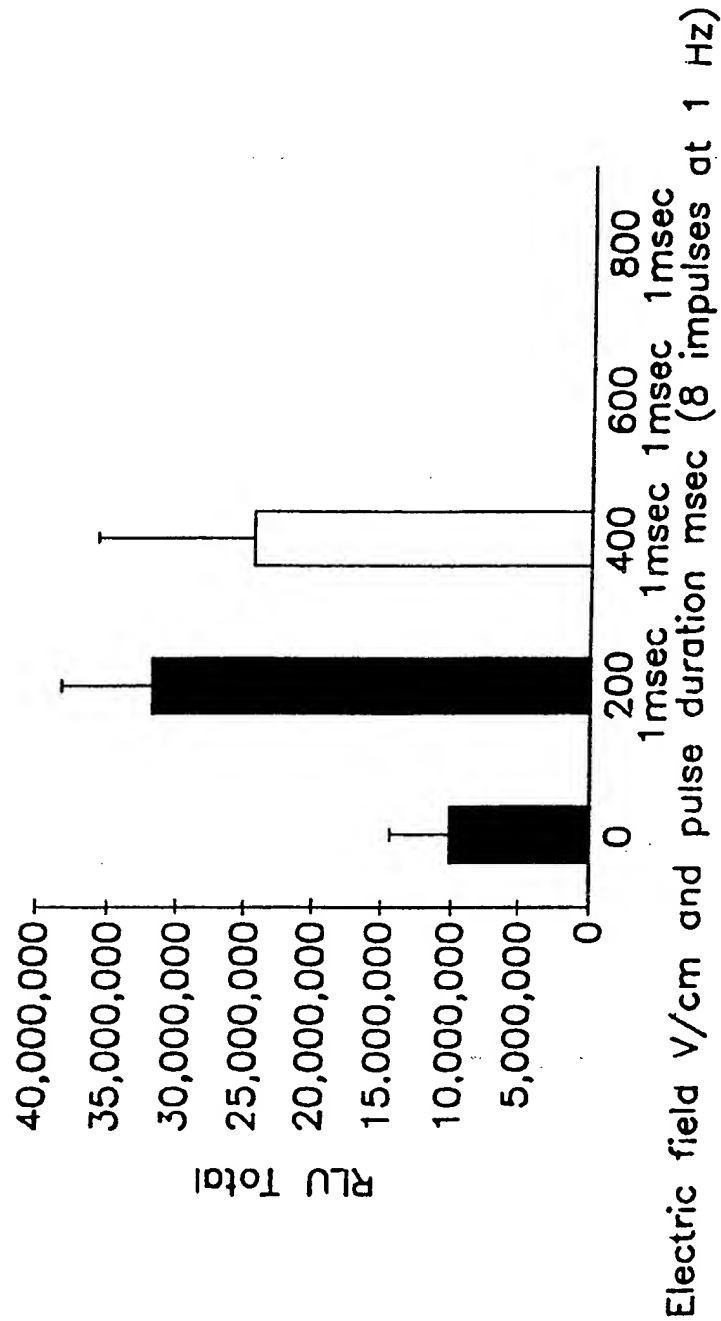


Figure 2b

Figure 3: Effects of electric impulses of weak field intensity and of different durations on the transfection of plasmid DNA p_{xl} 2774 in the cranial tibial muscle in the mouse. Mean values \pm SEM.

A. Logarithmic scale

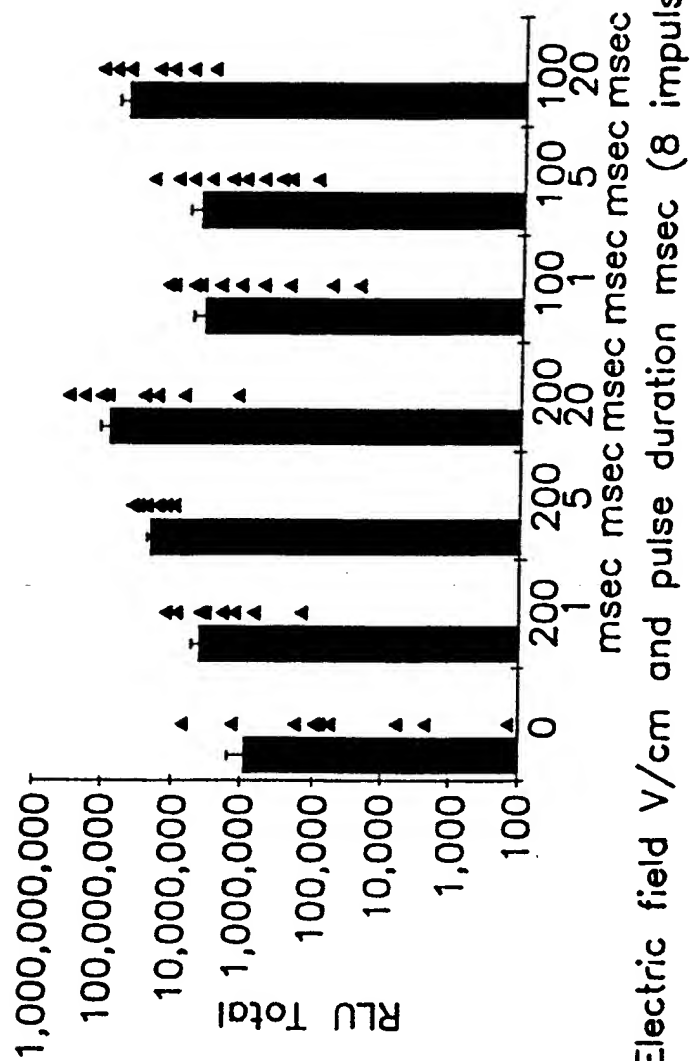


Figure 3a

Figure 3: Effects of electric impulses of weak field intensity and of different durations on the transfection of plasmid DNA p_{GL} 2774 in the cranial tibial muscle in the mouse. Mean values \pm SEM.

B. Linear scale

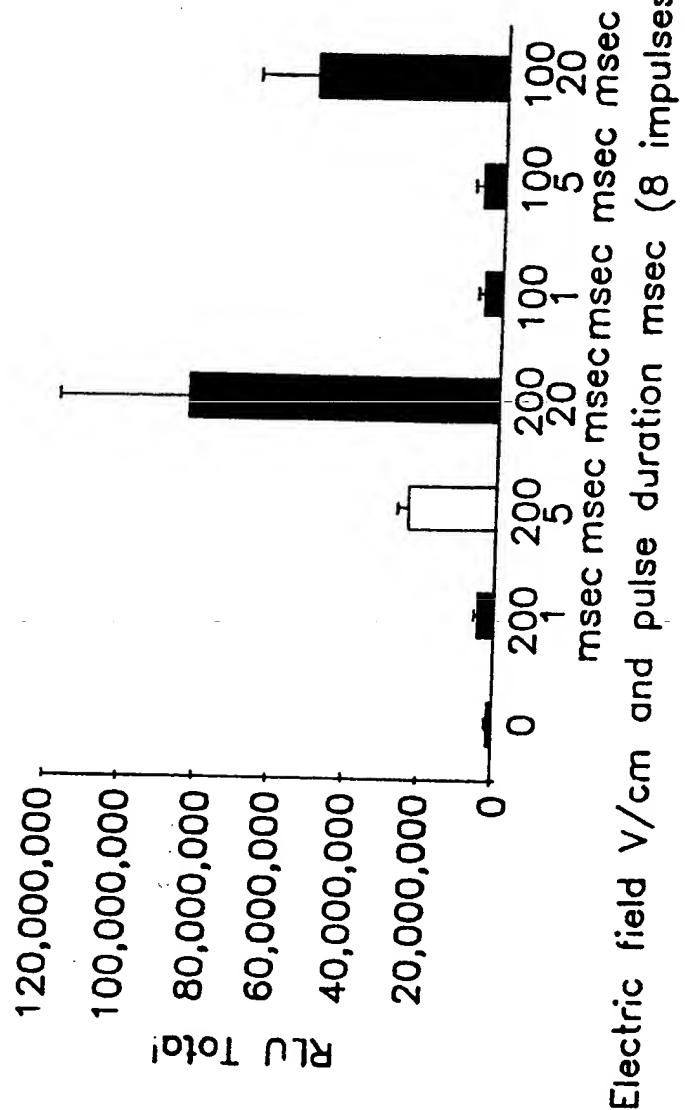


Figure 3b

Figure 4: Effects of electric impulses of weak field intensity and of different durations on the transfection of plasmid DNA p_{xl} 2774 in the cranial tibial muscle in the mouse. Mean values \pm SEM.

A. Logarithmic scale

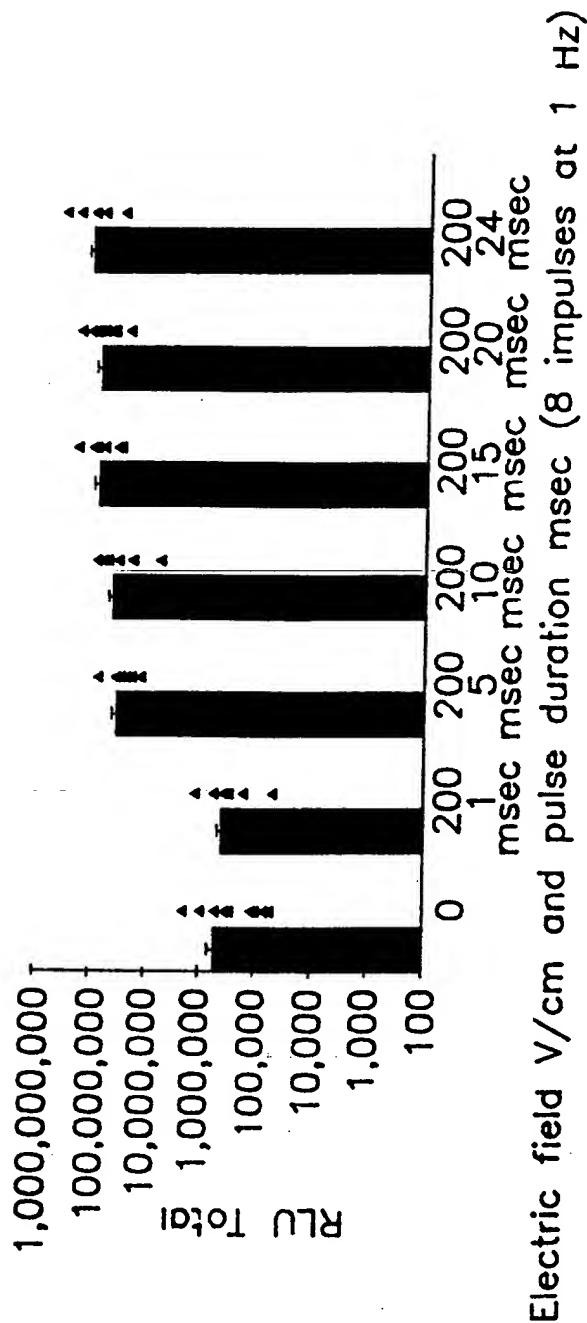


Figure 4a

Figure 4: Effects of electric impulses of weak field intensity and of different durations on the transfection of plasmid DNA p_{xl} 2774 in the cranial tibial muscle in the mouse. Mean values \pm SEM.

B. Linear scale

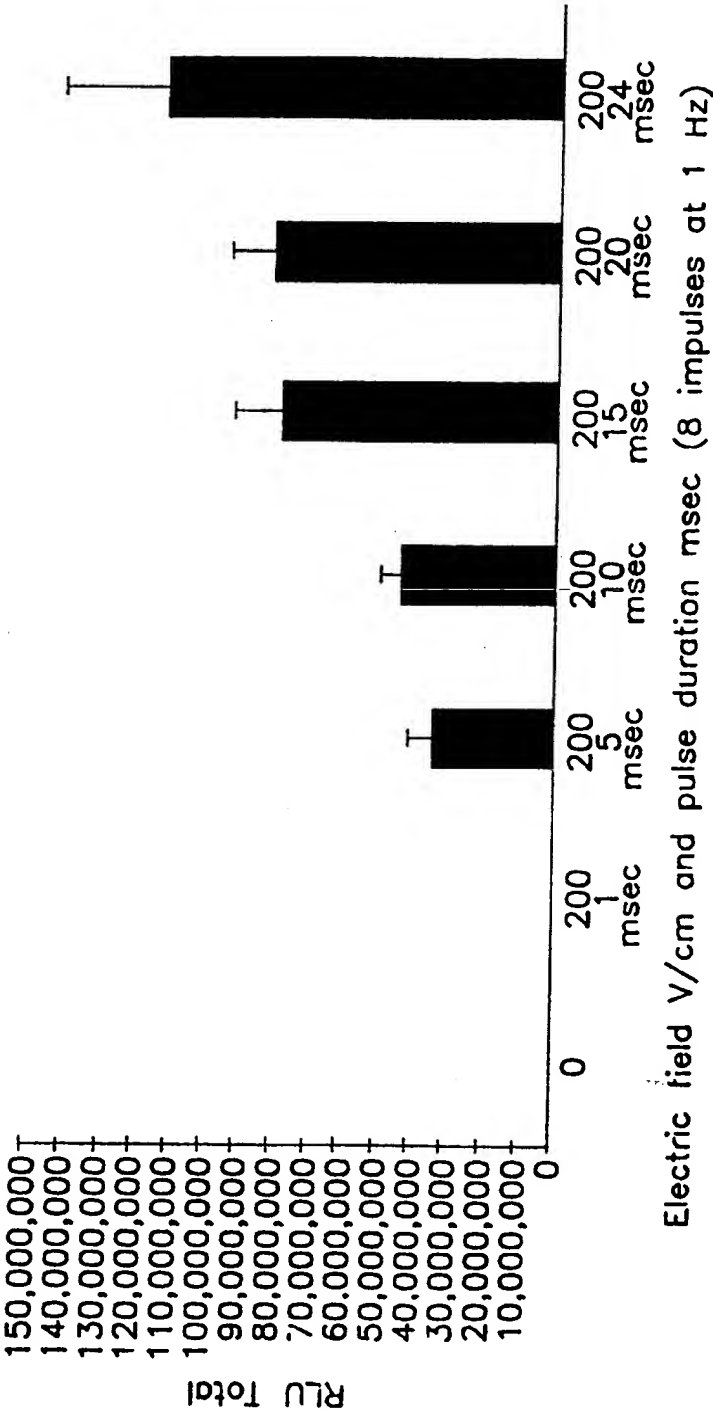


Figure 4b

Figure 5: Effectiveness of electrotransfection of plasmid DNA p_{xl} 2774 in the cranial tibial muscle of the mouse at low electric field intensities. Mean values \pm SEM.

Luciferase expression in relation to the mean value of the control group (DNA alone) which was standardized at 1.

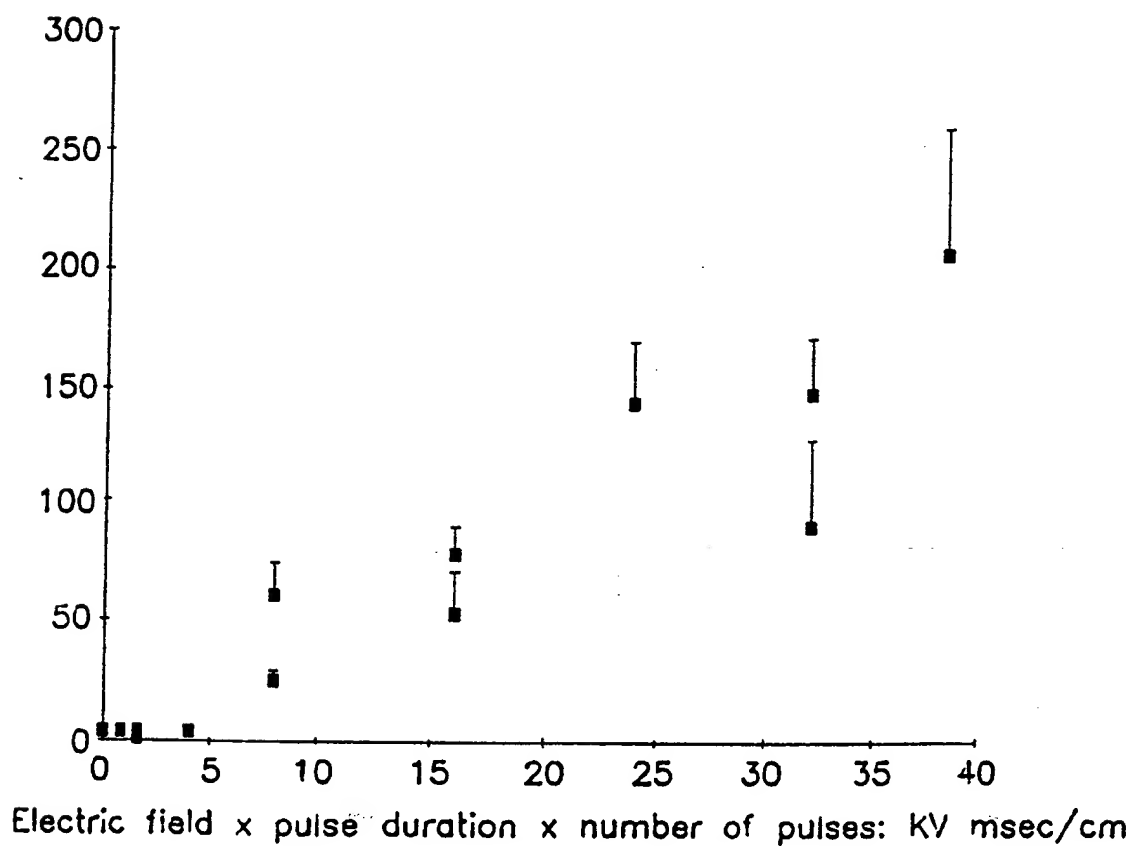


Figure 5

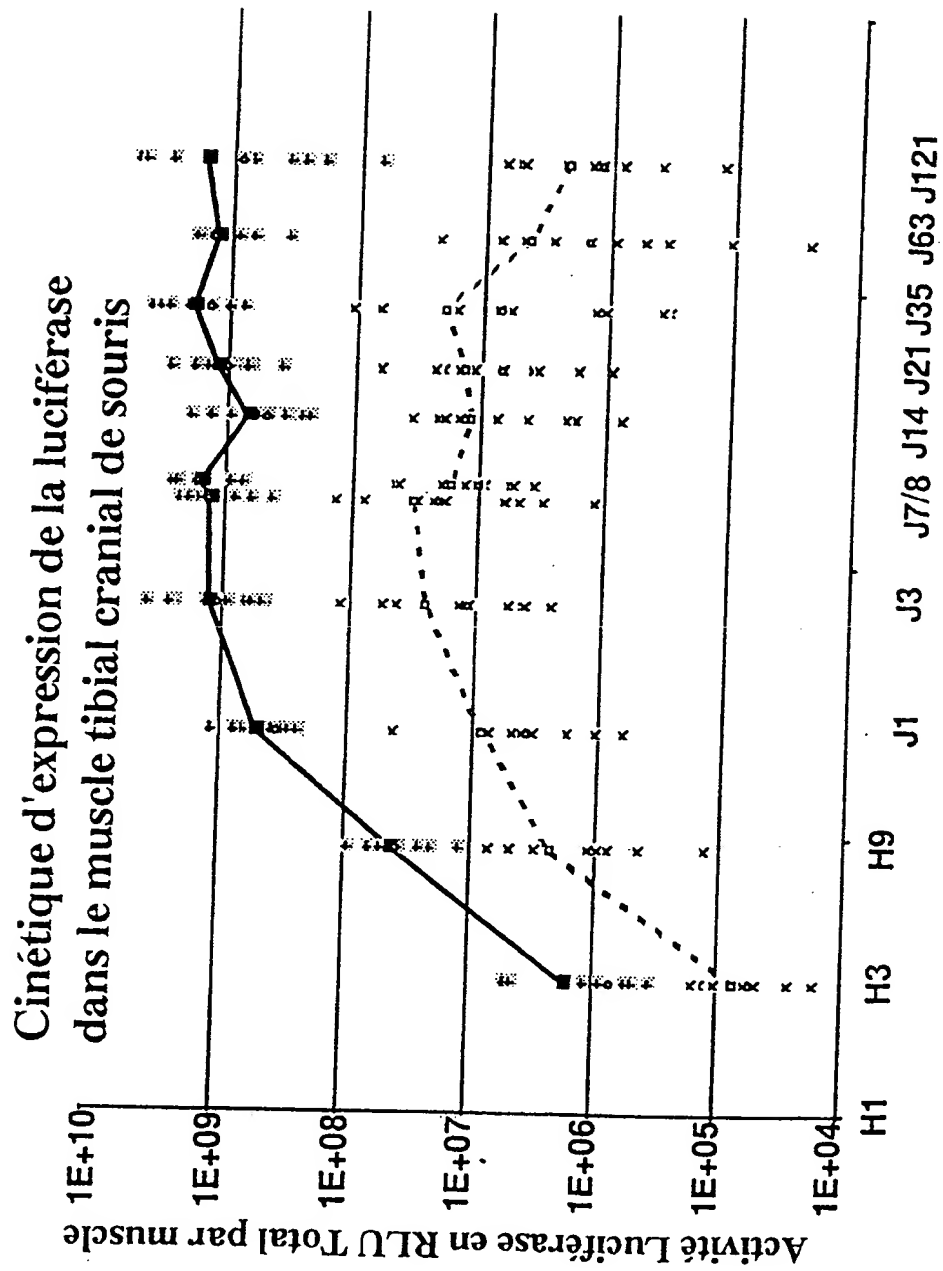


Figure 6

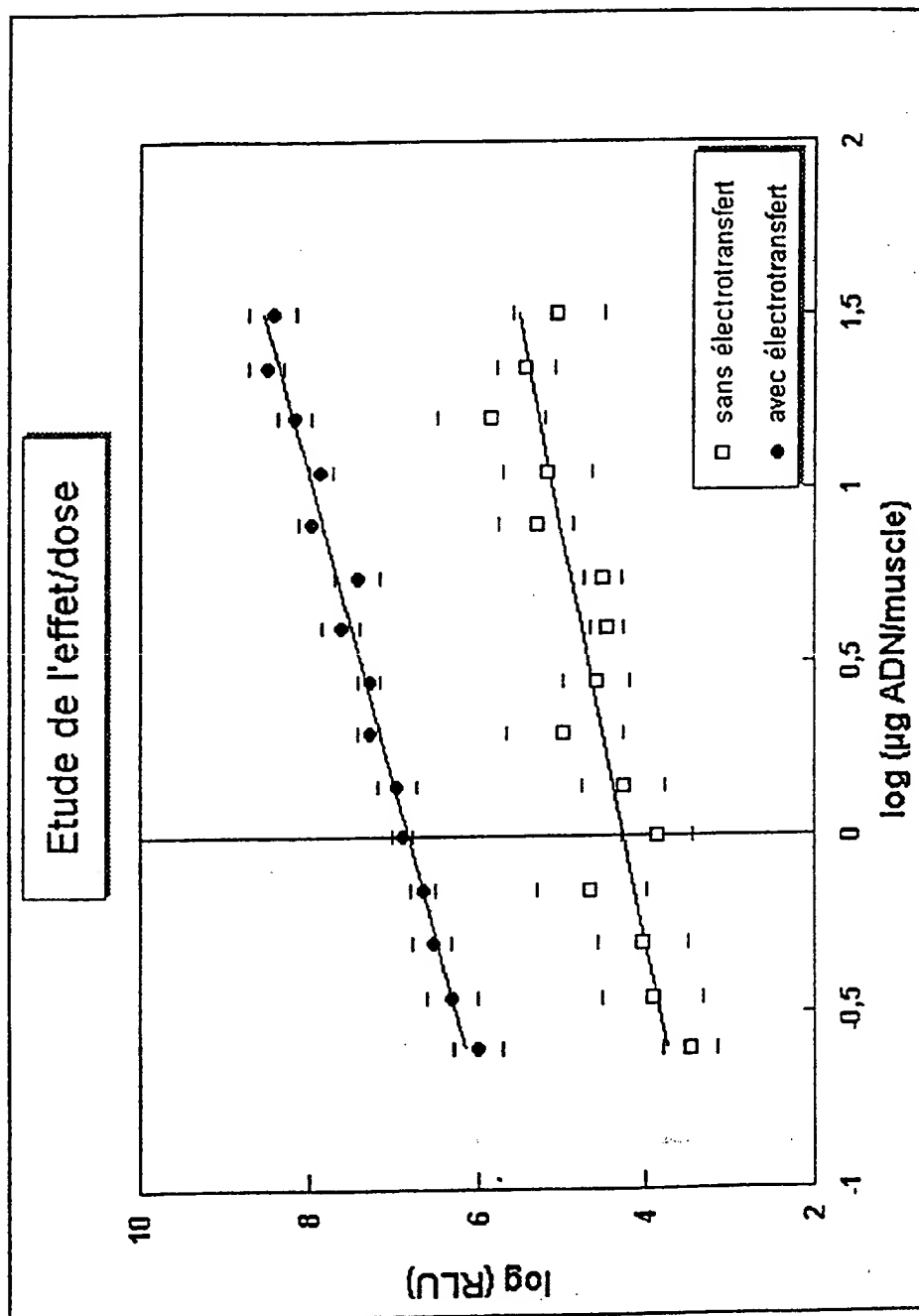


Figure 7

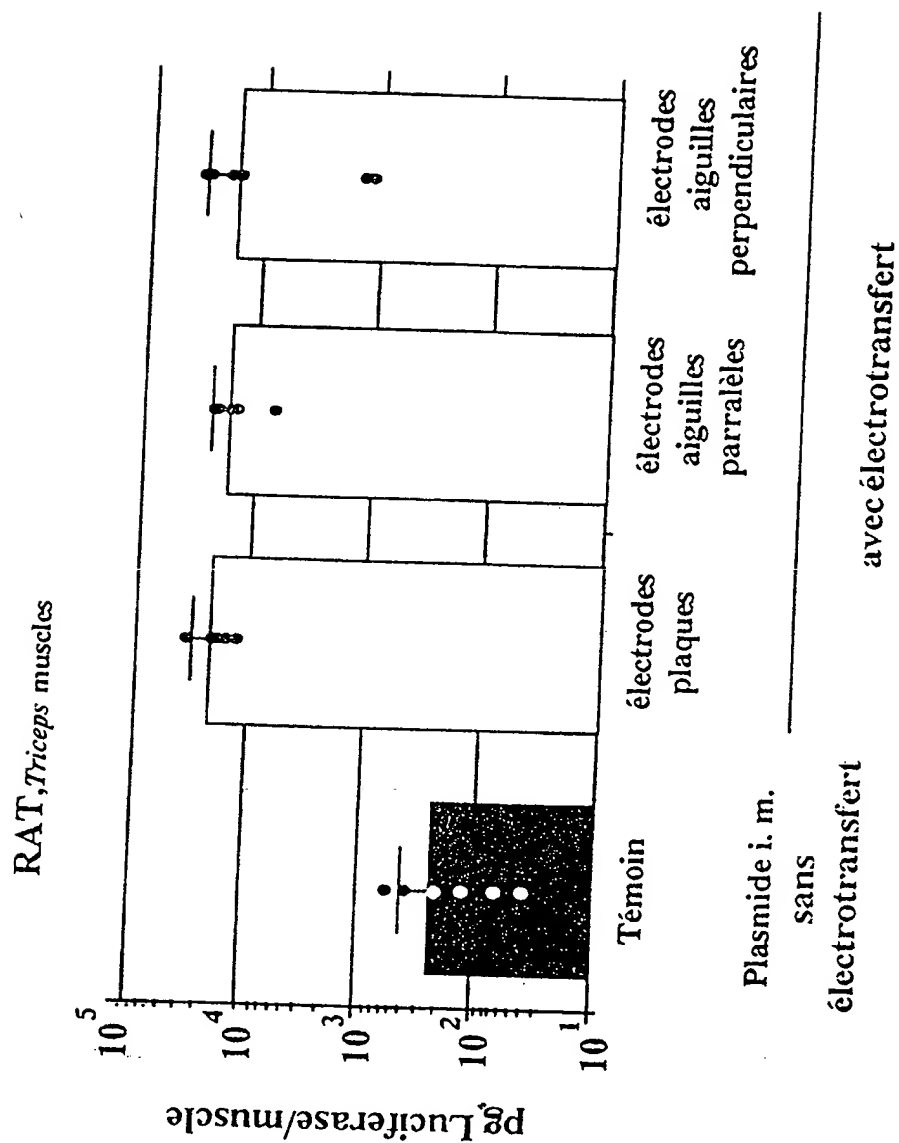


Figure 8

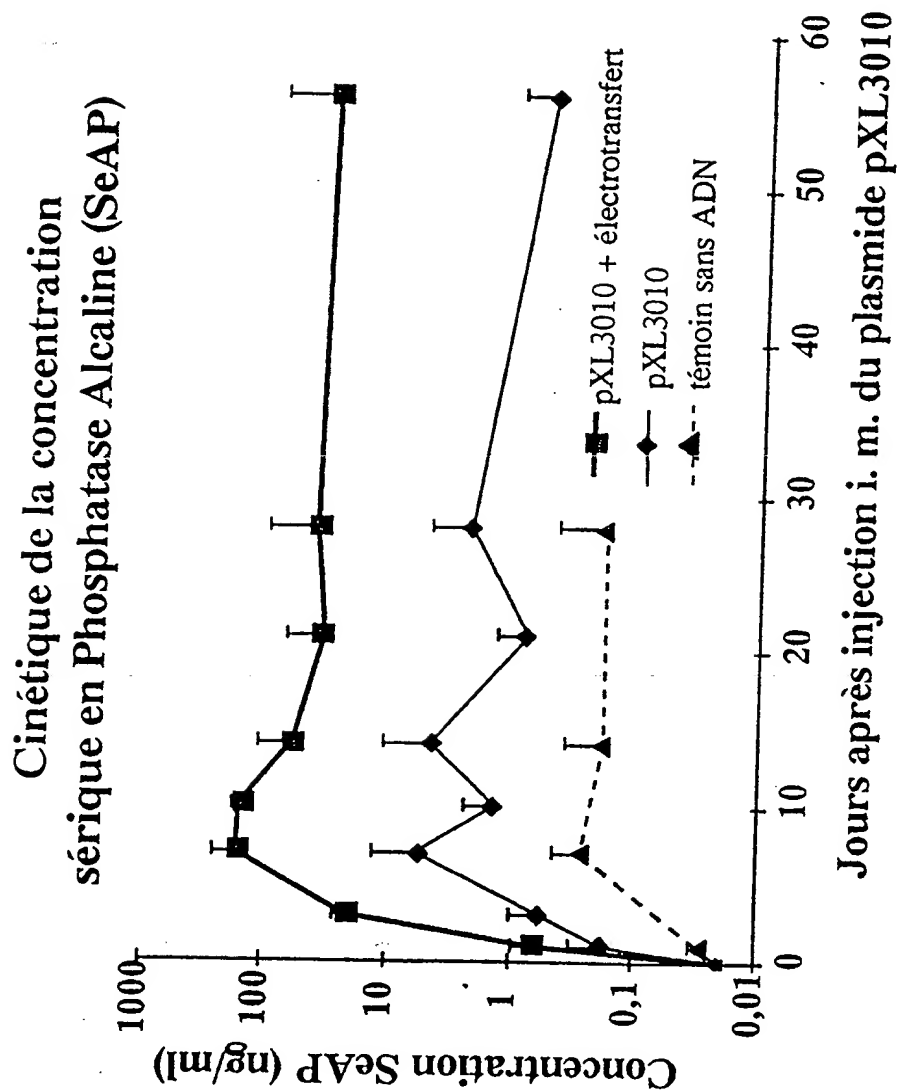
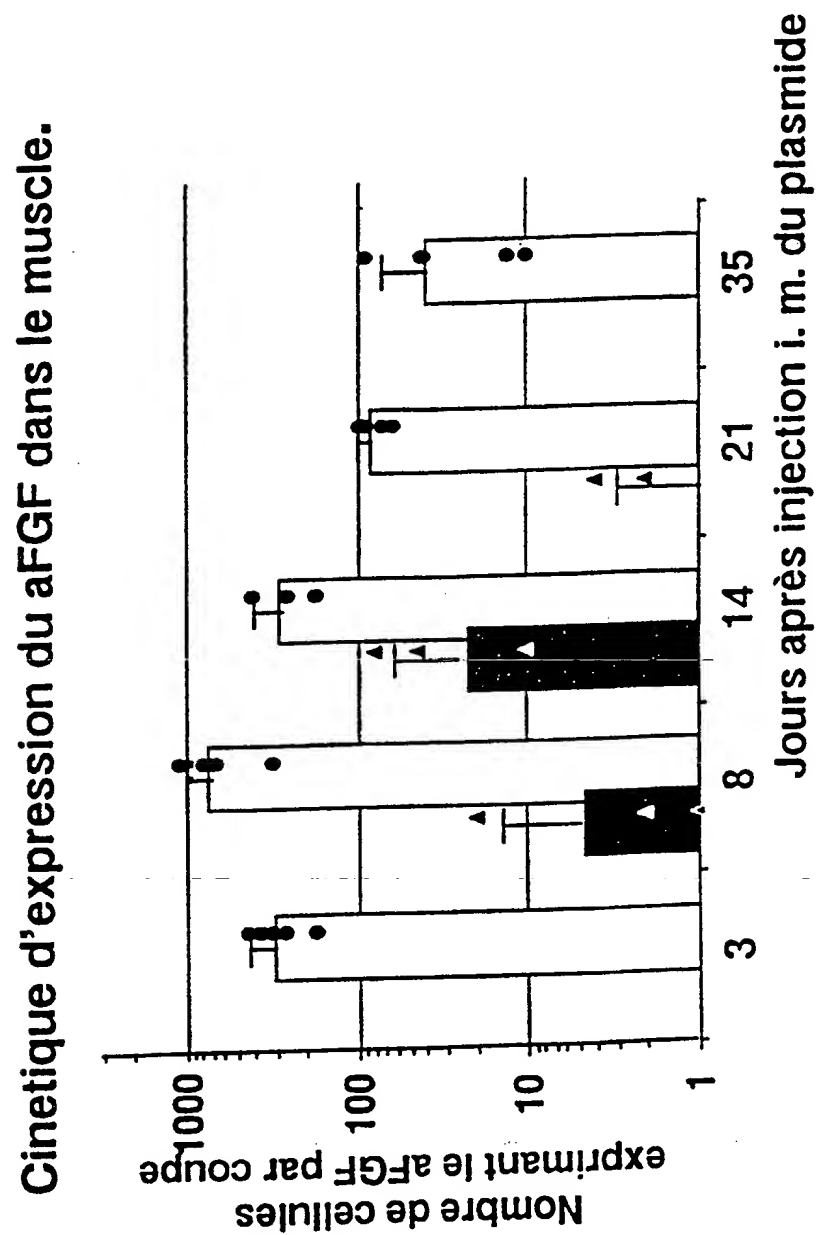


Figure 9



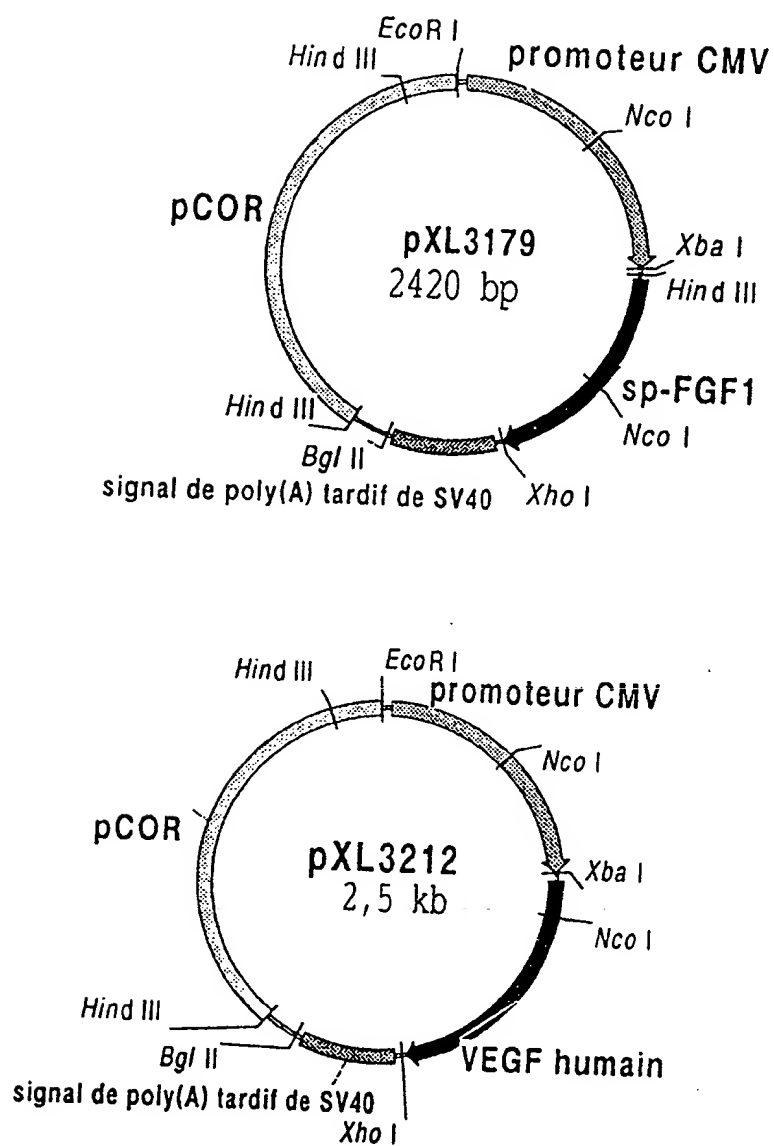


Figure 11

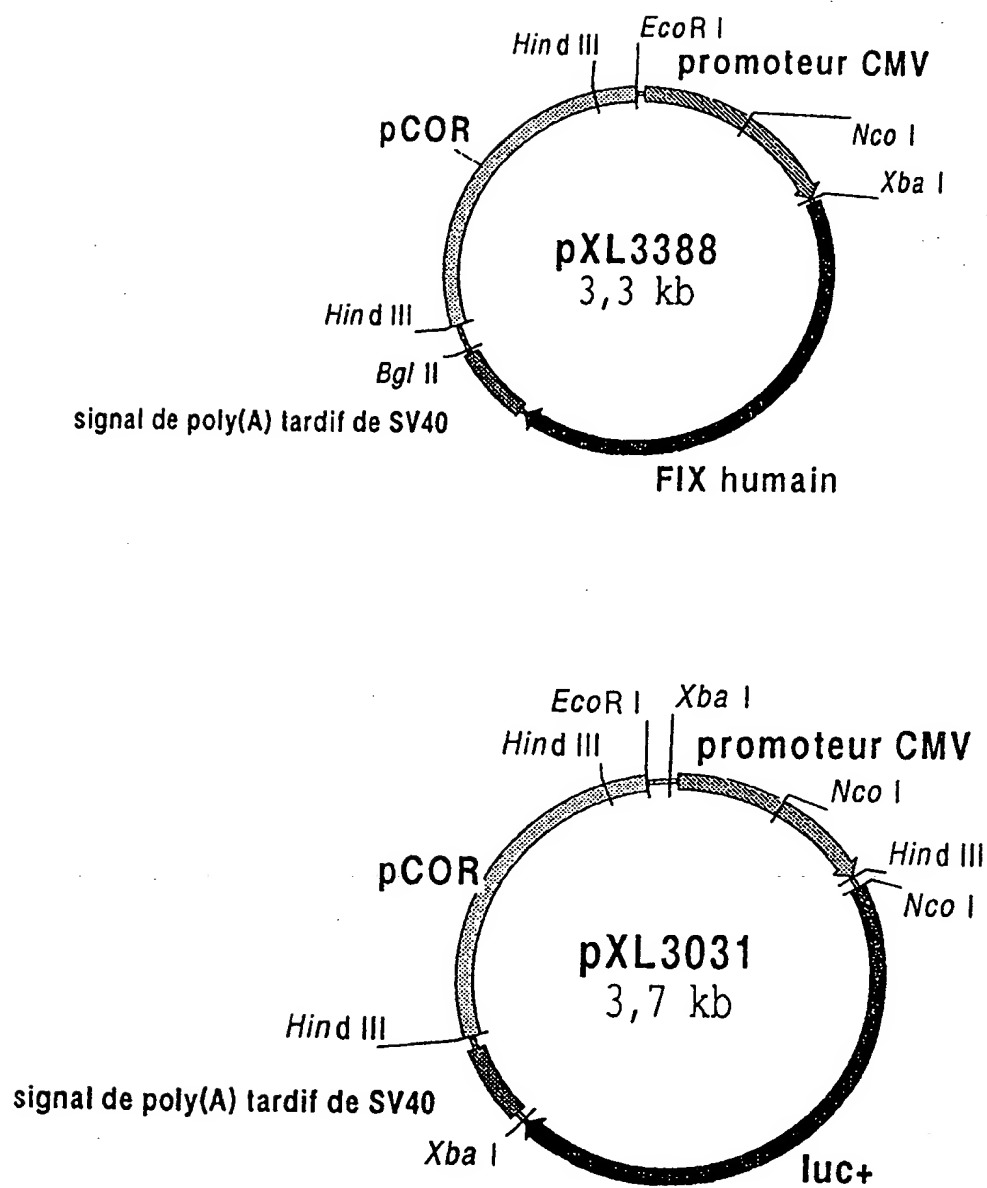


Figure 12

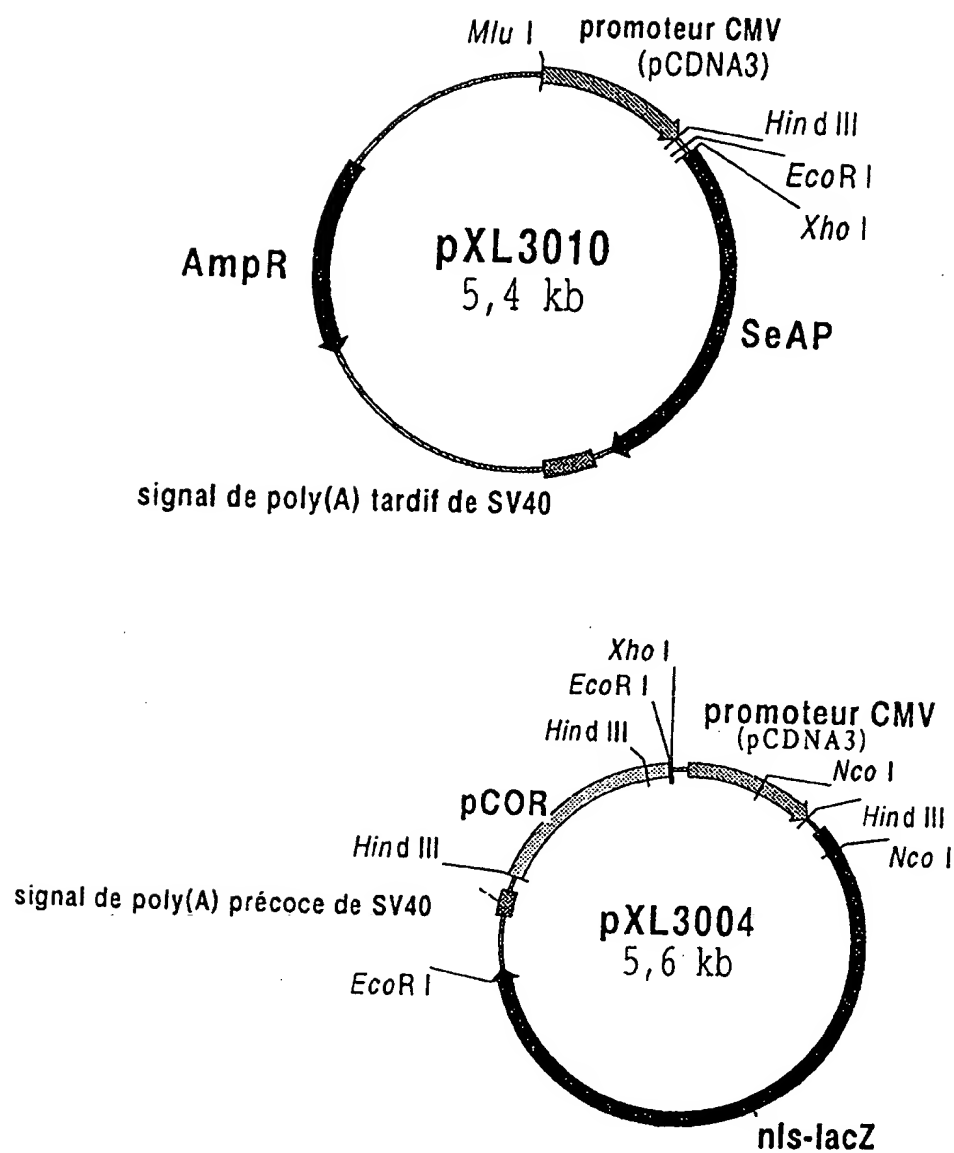


FIGURE 13

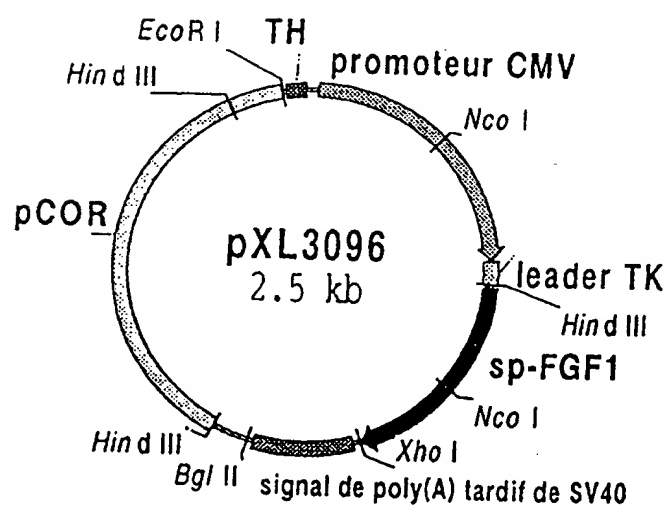
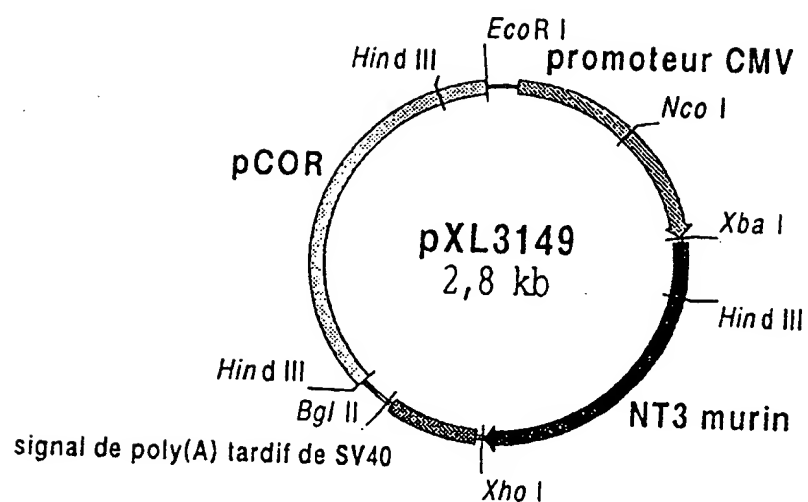


FIGURE 14

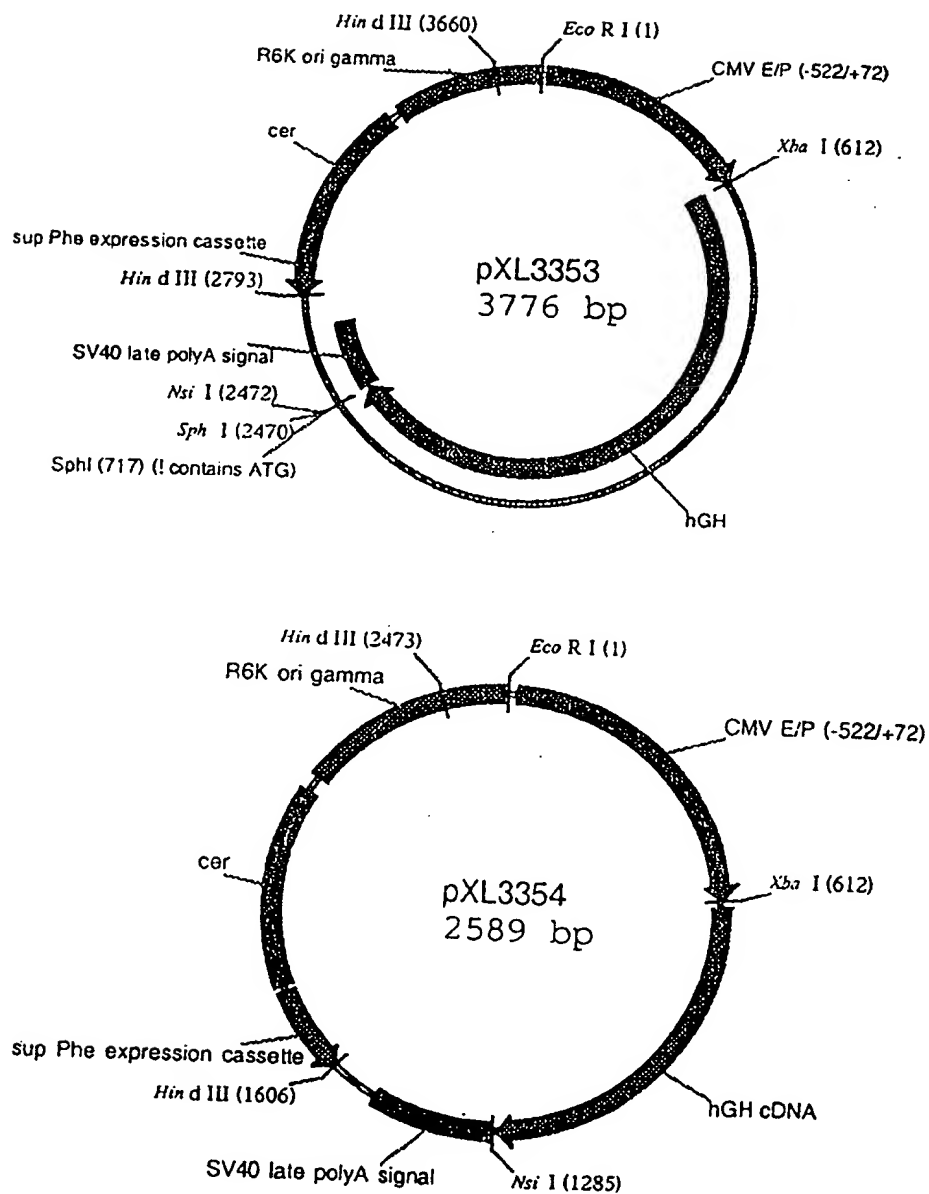


Figure 15

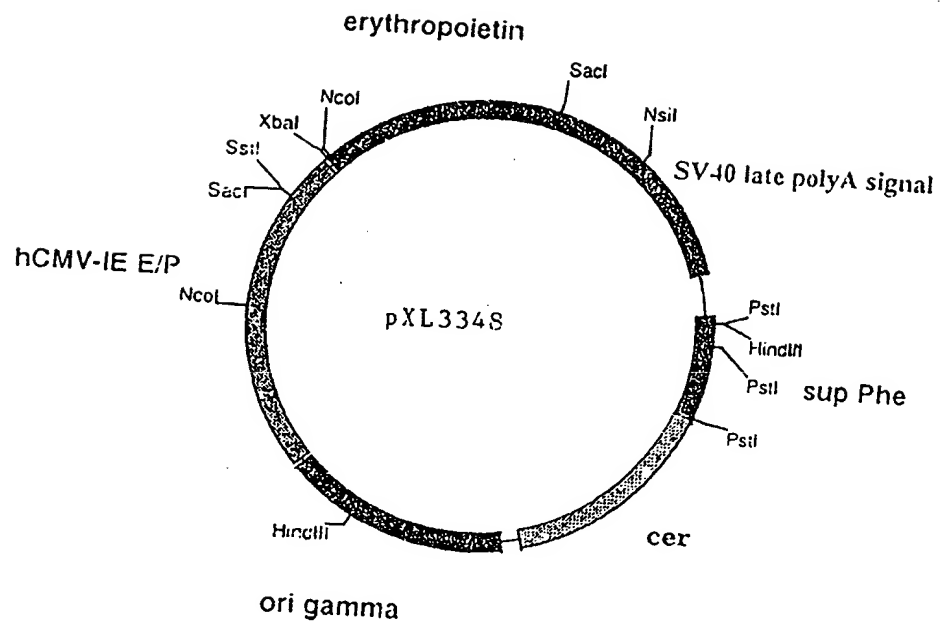


Figure 16

INTERNATIONAL SEARCH REPORT

Int. .ational Application No

PCT/EP 98/03976

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61N1/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 14836 A (INCELL) 23 May 1996 see page 4, paragraph 1 - page 7, paragraph 1 see page 10, paragraph 1 - page 22	1-37
X	WO 97 07826 A (CBR LAB INC ;UNIV SOUTH FLORIDA (US)) 6 March 1997 cited in the application see the whole document	1-37
X	WO 95 06129 A (THEREXSYS LTD ;KINGDON CRAIG R (GB); ANTONIOU MIKE (GB); DJEHA HAK) 2 March 1995 see the whole document	1-37
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

4 December 1998

Date of mailing of the international search report

14/12/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/03976

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HELLER ET AL: "IN VIVO GENE ELECTROINJECTION AND EXPRESSION IN RAT LIVER" FEBS LETTERS, vol. 389, 1996, pages 225-228, XP002058383 cited in the application see the whole document</p> <p style="text-align: center;">---</p>	1-37
X	<p>NISHI ET AL: "HIGH-EFFICIENCY IN VIVO GENE TRANSFER USING INTRAARTERIAL PLASMID DNA INJECTION FOLLOWING IN VIVO ELECTROPORATION" CANCER RESEARCH, vol. 56, 1996, pages 1050-1055, XP002058384 see the whole document</p> <p style="text-align: center;">-----</p>	1-37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/03976

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614836 A	23-05-1996	US 5641680 A AU 4138196 A	24-06-1997 06-06-1996
WO 9707826 A	06-03-1997	AU 6855796 A	19-03-1997
WO 9506129 A	02-03-1995	AU 7465194 A CA 2169828 A EP 0725832 A JP 9504425 T US 5766902 A	21-03-1995 02-03-1995 14-08-1996 06-05-1997 16-06-1998